EFFECTS OF FLOCCULATION ON RETROVIRUS PROCESSING, DELIVERY AND TRANSDUCTION

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EFFECTS OF FLOCCULATION ON RETROVIRUS PROCESSING, DELIVERY AND TRANSDUCTION

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To my Mother and to my Sister

To Mario

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SUMMARY

Gene therapy consists of the intracellular delivery of genetic material to produce a therapeutic effect. One of the most common means used for transferring genes to cells are recombinant retroviruses because they infect a variety of cell types and they stably integrate the therapeutic gene into the genome of the target cell, resulting, in principle, in a long-term cure. Unfortunately, their success has been limited, in part because they fail to transfer enough genes to achieve a desired biological effect. In addition, it has proven difficult to achieve predictable and reproducible levels of gene transfer using this vector. Some of the major limitations of retroviral-mediated gene transfer that are responsible for low and variable levels of gene transfer have been identified. These limitations include the low probability of encounter between bioactive viruses and target cells, the presence of inhibitors of transduction in virus stocks, and the inefficient interactions between viral envelope proteins and their cognate cellular receptors.

We developed a method that substantially enhances the efficiency of retroviral-mediated gene transfer. The method consists of the combined addition of the cationic polymer Polybrene and the anionic polymer chondroitin sulfate C to retrovirus stocks prior to transduction. These polymers induced flocculation of virus particles into high molecular weight retrovirus-polymer complexes that incorporated more than 80% of the viruses and less than 0.3% of the proteins originally present in the stock. The complexes were not cytotoxic, did not reduce the stability of the viruses, and were large enough to sediment, delivering the viruses to the cells more rapidly than by simple diffusion. Increases in the rate of transport of viruses correlated with increases in the rate of transduction, as the polymers did not affect the efficiency of post-binding steps of transduction.

The formation of virus-polymer complexes not only permitted accelerating the transport of viruses to cells, but also enabled concentrating virus stocks. Low speed centrifugation of the complexes for 5 min, followed by resuspension of the pellet in a reduced volume resulted in substantial enhancement of transduction. Additionally, given that the complexes selectively incorporated virus particles, we were able to purify stocks from inhibitors of transduction by removing the conditioned medium and resuspending the pellet in fresh cell culture medium. Purification increased the maximum level of gene transfer up to 186-fold and linearized the dose response curve, thereby simplifying attempts to obtain reproducible and predictable levels of transduction.

When using high concentrations of purified viruses, the efficiency of gene transfer was dependent on the number of envelope proteins displayed on the surface of each virus particle. Viruses with a low number of envelope proteins transduced cells more efficiently than did viruses with a high number of envelope proteins, and allowed more integrations of the transgene per target cell. In contrast, when the number of envelope proteins per virus particle was high, transduction appeared to be limited by a reduction in availability of functional receptors for viruses pseudotyped with the same envelope.

In summary, we have shown that the efficiency of retroviral-mediated gene transfer can be dramatically enhanced by inducing flocculation of retroviruses into polyelectrolyte complexes. The complexes sediment onto cells, can be rapidly concentrated without inactivating the virus particles, and can be purified from inhibitors. Purified virus stocks can be used in smaller quantities than unprocessed stocks to achieve a desired level of gene transfer and reduces uncertainties about the relationship between the amount of virus used and the number of genes transferred. In addition, the efficiency of gene transfer, and potentially the number of transgene copies delivered per target cell, can be modulated by varying the number of envelope proteins that protrude

from a virus particle. Taken together, these novel methods for processing retrovirus stocks and a better understanding of major limitations of transduction should simplify efforts to predict the outcome of retrovirus transduction protocols and should help to increase the likelihood that human gene therapy protocols will succeed.

CHAPTER 1

BACKGROUND AND OBJECTIVES

1.1 Gene Therapy

Gene therapy is the intracellular delivery of genetic material to achieve a therapeutic effect. It was initially conceived to treat simple inherited genetic disorders, but has since been expanded to a wide range of applications. Gene therapy is currently under investigation to treat complex acquired genetic disorders such as cancer and infectious diseases such as HIV, and has numerous applications in tissue engineering [1]. Unfortunately, in spite of the enormous potential that treating diseases at the genetic level holds, there have been relatively few successes to date in gene therapy clinical trials, largely due to limitations with the current generation of gene transfer vectors. Gene transfer vectors can be classified as viral and non-viral [1, 2]. Viral vectors are replication-incompetent derivatives of wild-type viruses that take advantage of the mechanism viruses have developed over millions of years to deliver genes in an efficient manner and that have been genetically engineered to deliver therapeutic genes to cells. Non-viral vectors include all other types of gene transfer vectors and typically involve the delivery of genetic material to cells as part of polymer or liposomal complexes or via physical methods (by disrupting the cell membrane by mechanical or electrical means).

The delivery of genetic material for therapeutic applications can be conducted either *in vivo* or *ex vivo*. In the *in vivo* setting, gene delivery vectors are directly injected into the patient. In the *ex vivo* setting, tissue is removed from the patient, cells from this tissue are cultured *in vitro*, genetically modified, then selected, expanded and reimplanted back into the patient. The *ex vivo* delivery of genes allows for relatively

straightforward targeting of gene delivery vectors and optimization of the gene transfer conditions. However, this method is labor intensive, expensive and not applicable to tissues that cannot be removed and reimplanted (e.g. brain). On the other hand, *in vivo* delivery requires less manipulation of the tissue, but also requires specific targeting to the cells of interest.

1.2 Recombinant retroviruses as gene delivery vectors

Currently, recombinant retroviruses are the vector of choice for many gene therapy applications because they are able to permanently integrate their genome into the genome of the target cell, resulting, in principle, in a long-term cure [3, 4]. Retroviruses used for gene transfer are often derived from the Moloney murine leukemia virus (MLV) or from the human immunodeficiency virus type 1 (HIV-1), which are also classified as lentiviruses. The most significant functional difference between these two viruses is that recombinant retroviruses delivered from MLV can only infect cells that are actively dividing, whereas recombinant lentiviruses derived from HIV may be able to infect cells even if they are not dividing [5, 6], an important advantage for many *in vivo* gene therapy protocols [7, 8].

Recombinant retroviruses for gene therapy are designed to adopt the natural ability of wild type retroviruses to integrate into the genome of the target cells. Wild-type retroviruses are composed of two copies of identical strands of genomic RNA that are enclosed within an icosahedral matrix of virus capsid proteins. The capsid proteins are in turn surrounded by a lipid bilayer membrane, from which envelope glycoproteins protrude. Recombinant viruses are structurally identical to wild-type viruses, but carry a genetically engineered genome (vector), which encodes for the therapeutic gene of interest and does not encode for viral genes required for replication. Due to this design,

recombinant retroviruses deliver genes to cells in a similar manner to that of wild type viruses, but are not able to self-replicate.

Recombinant retroviruses are produced by a two-part system composed of a packaging cell line and a recombinant vector (Figure 1.1). The packaging cell line is engineered to express all the viral genes (gag, env and pol) required for the formation of infectious virus particles. The retroviral vector contains the gene of interest, regulatory sequences for gene expression and a packaging sequence (ψ) for recognition and packaging of the viral RNA genome in a virus particle. During virus production, packaging cell lines are incubated with cell culture medium for several hours. During this time, they express the viral proteins and transcribe the viral vector. The viral proteins recognize the ψ packaging sequence of the vector RNA and assemble it into viral particles that bud from the cell membrane. The virus-laden medium is then collected and used to transfer genes to target cells.

The process of gene transfer (transduction) consists in incubating virus stocks with target cells and requires the completion of a series of complex steps that begins with the transport of the virus particles to the surface of the cells. Once a virus binds to the surface of a cell, envelope proteins that protrude from the virus specifically bind to cellular receptors. This triggers fusion of the lipid bilayers of the virus and the cell. Upon fusion, the RNA genome of the virus and associated virus proteins and enzymes are released to the cytosol of the cell. After the virus enters the cytosol, the RNA genome of the virus is reverse transcribed to DNA, which is transported to the nucleus and integrated into the chromosomal DNA of the host cell. Once integrated, the viral DNA is stable and inherited by daughter cells, and its expression leads to the synthesis of the transgene (Figure 1.2).

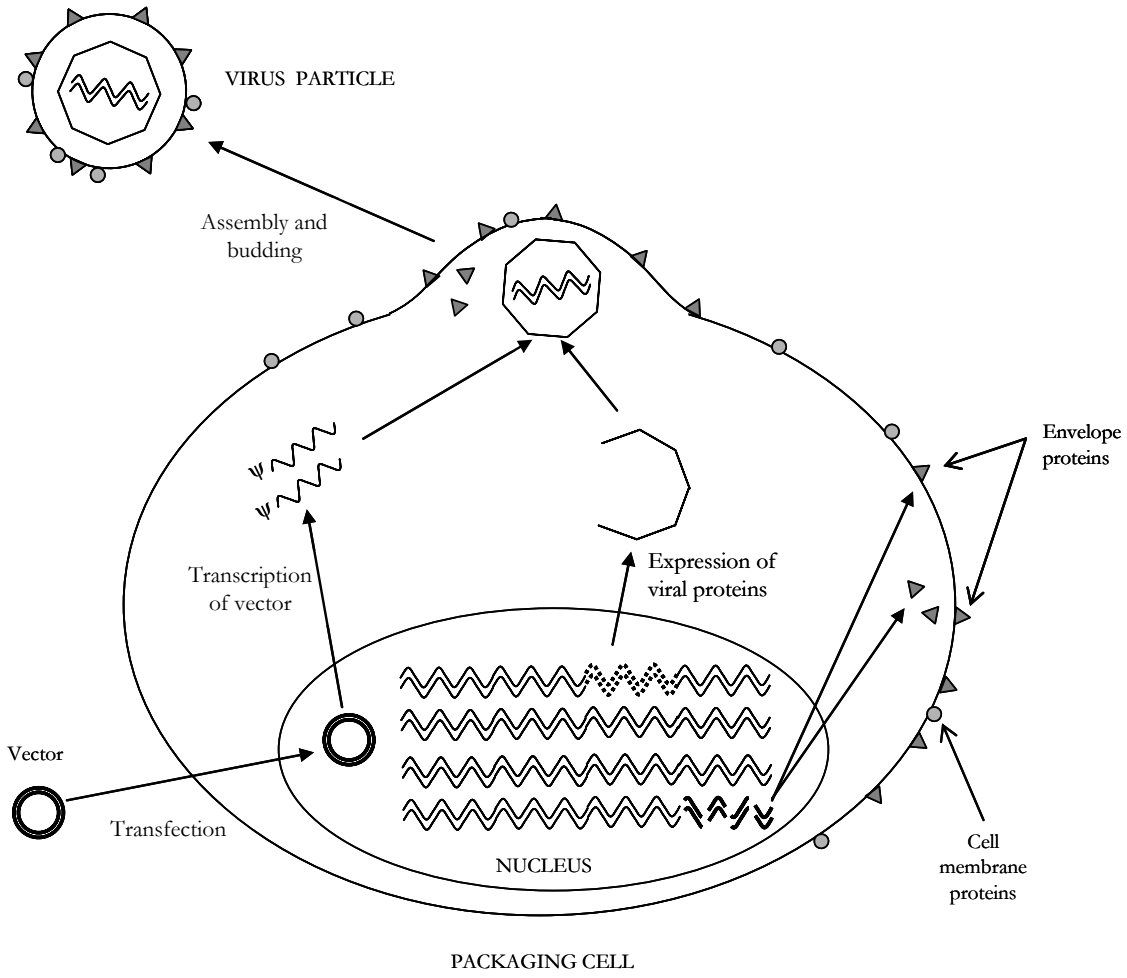

Figure 1.1 Production of recombinant retroviruses by packaging cell lines. Recombinant retroviruses are produced by transfecting packaging cells, genetically modified to express all of the proteins necessary to form a retrovirus particle, with plasmid DNA that encodes the therapeutic gene(s) of interest, the regulatory sequences that drives their expression, and a packaging sequence that ensures the RNA genomes of the viruses are efficiently incorporated into the virus particles. The virus capsid proteins self-assemble around the RNA genomes that contain the packaging sequence, then bud from the surface of the cell into the surrounding cell culture medium. In the process of budding, the virus particles incorporate a lipid bilayer, which contains the virus envelope proteins and other 'passenger' cellular proteins, from the plasma membrane of the cell.

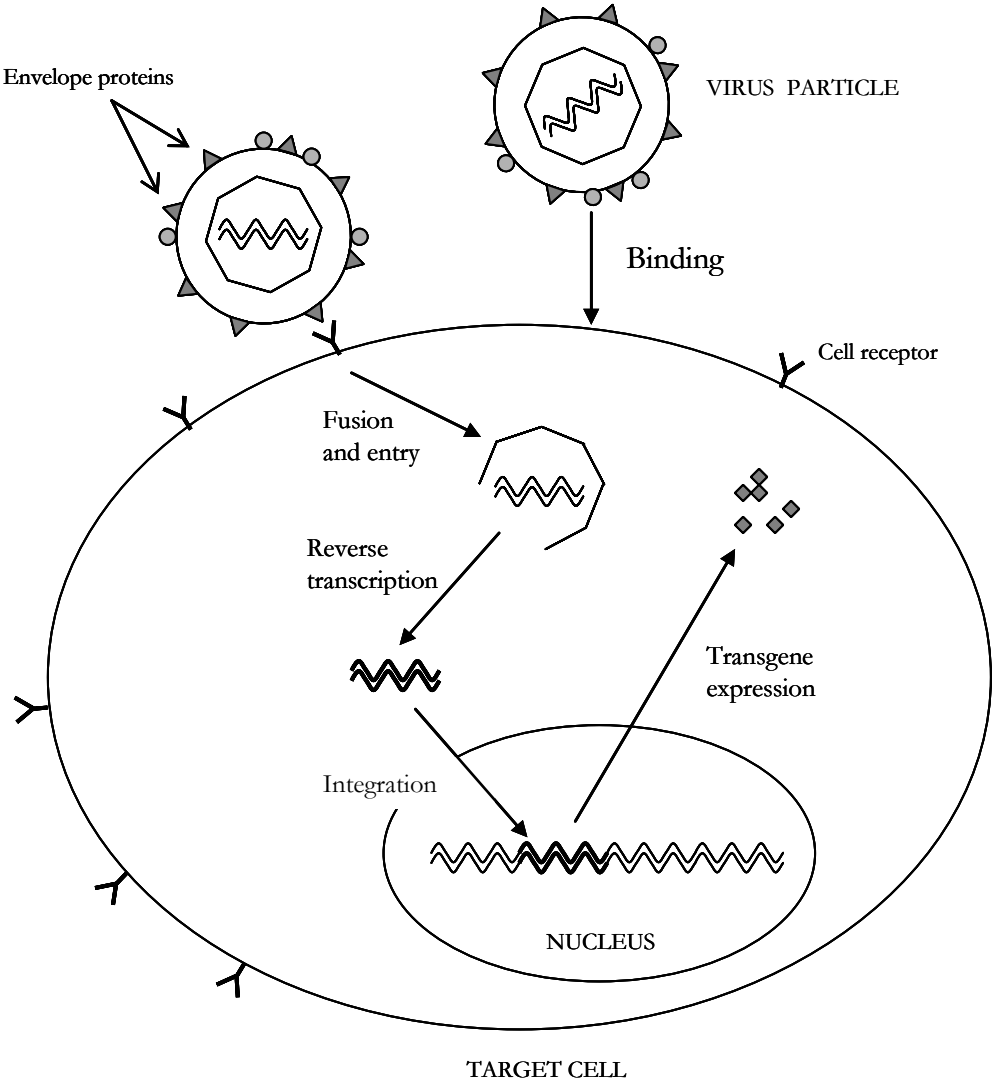

Figure 1.2 Transduction of target cells by recombinant retroviruses. Cells are transduced by incubation with stocks of retroviruses. Retroviruses diffuse to the surface of the cells, and then bind to them via poorly defined non-specific interactions between the surface of the virus and the cell. The envelope proteins of the virus then interact with their cognate receptors on the surface of the cell. This interaction initiates fusion between the lipid bilayer of the virus and the plasma or endosomal membrane of the cell, and the subsequent release of the internal components of the virus to the cytosol. In the cytoplasm, the RNA genomes of the viruses are reverse transcribed to DNA, then transported to the nucleus and integrated into the chromosomal DNA of the cell. The therapeutic gene(s) are then expressed.

1.3 Limitations of retroviral-mediated gene transfer

Unfortunately, the use of recombinant retroviruses in the clinic has met with limited success, in part because retroviruses often transfer too few genes to cells to achieve a desired therapeutic effect [9]. In addition, it has proven difficult to achieve predictable and reproducible levels of gene transfer with retroviruses [10]. Some of the major limitations of retroviral-mediated gene transfer that are responsible for low and variable levels of gene transfer have been identified. In this section we will describe major limitations, as well as strategies that have been developed to overcome them.

Low probability of encounter between infectious viruses and target cells

The low probability of encounter between bioactive viruses and cells is due to the combined effect of rapid decay and slow transport of retrovirus particles. Retroviruses lose their activity with a half-life between 6 and 8 hours [11, 12] and their transport in solution is driven by diffusion, with a diffusion coefficient of 2×10^{-8} cm^2/s [13]. Considering these parameters, it has been estimated that in a typical gene transfer protocol, just 10% of the total number of virus particles are able to diffuse, bind and successfully transduce cells before losing their bioactivity [12]. Binding of viruses to cells is the first interaction necessary for transduction. It does not require specific binding of viral envelope proteins to receptors on the surface of target cell as previously thought [14-17], but is mediated by non-specific interactions. Thus, efforts to enhance the efficiency of gene transfer have focused on developing methods to increase the rate of delivery of infectious viruses to the surface of the cells. These methods include centrifugation of the virus stocks over target cells, flow through filtration, tangential flow filtration, complexation of virus particles with agents that lead to sedimentation of viruses onto target cells, addition of low concentrations of cationic polymers, and coating of the tissue culture plate with fragments of fibronectin (where both viruses and cells appear to

rapidly bind and colocalize) [18-26]. These methods can only be used *ex vivo* and increase the concentration of viruses on the surface of the target cells.

Other strategies to increase the number of binding events between infectious viruses and target cells have focused on increasing the concentration of virus particles in the stocks (Table 1.1). Concentration has been conducted by ultracentrifugation, low speed centrifugation (usually for several hours), ultrafiltration, incorporation of viruses into complexes that are easily pelleted, and association of viruses with paramagnetic particles that are concentrated using a magnetic field [24, 27-39]. These methods can be used to process virus stocks prior to transduction *ex vivo* or *in vivo*.

Although most of these approaches have resulted in significant increases in gene transfer, often times fold enhancements have been lower than those expected. This can be in part explained by the presence of inhibitors of transduction, which are secreted by packaging cell lines as a byproduct of the virus production system [40, 41].

Presence of inhibitors in virus stocks

During virus production, packaging cell lines secrete a variety of molecules into the cell culture medium, some of which act as inhibitors of transduction. The nature and concentration of these inhibitors depend on the producer cell line used. As a result, the inhibitory activity can substantially differ from stock to stock of the same virus [42]. Some of these inhibitors have been identified and include proteoglycans, glycosaminoglycans and free envelope proteins [41-43].

Glycosaminoglycans are long repeating linear anionic polymers of disaccharides, which can be covalently bound to a central protein core to form proteoglycans. The extent of inhibition by glycosaminoglycans is independent of the envelope of the virus [40, 44] and dependent on the charge of the polymer, its degree of sulfation and its specific carbohydrate structure [40, 43]. The mechanism of inhibition is not fully

Table 1.1 Methods for concentration of virus stocks

Concentration Method	Virus	Starting volume (mL)	Fold volume reduction	Fold increase in transduction	Recovery by titer (%)	Recovery of virus particles	Time required	Cytotoxicity	Tested	Purification from inhibitors/other?	Details	Ref
ULTRAFILTRATION [a]												
Hollow fiber filtration	Amphotropic retrovirus	600-770	13-40	Lower than fold increase in titer	54-86	NM	2-6 hr	NM	In vitro	NM	500 kDa membrane, 2.5psi	[34]
Tangential flow filtration	Retrovirus	8000-10000	16-25		91-96	NM	30 min	NM	In vitro	NM	300 kDa membrane, 5 psi.	[25]
Ultrafiltration	Retrovirus	100-150	50-75		20-55	NM	2.5 hr	none	In vitro	NM	100 kDa membrane. Nitrogen pressure: 500 kpa	[33]
Ultrafiltration	VSVG, amphotropic and rabies-G lentivirus	60	61-69	NM	113-536	NM	~2-2.5hrs	NM	In vitro	May have removed an inhibitor	Centrifugation 1500 rpm (Beckman GPR, 2)-2.5 hours	[45]
LOW SPEED CENTRIFUGATION [b]												
Centrifugation	Amphotropic retrovirus	36	12-36	2-3 [e]	~50	NM	2 hr	NM	In vitro		15000 rpm (Beckman SW28), 2 hours, 4°C	[46]
Centrifugation	Amphotropic retrovirus	500	10-100	~13-20 (in vivo)	90-97	NM	16 hr	Animal mortality at highest concentrations of virus	In vitro/ In vivo	Albumin and other proteins in concentrate	6000g, 16 hours, 4°C	[27]
Centrifugation	Amphotropic and ecotropic retrovirus	NM	10-100	3-4 [g]		100% (viral RNA)	4 hr	NM	In vitro	NM	20000g, 4 hours, 4°C	[37]
Centrifugation	RD114-retrovirus		100	NM	37-80	NM	24 hr	NM	Ex vivo (implant)	NM	7277g, 24 hours, 4°C	[47]
HIGH SPEED CENTRIFUGATION [c]												
Ultracentrifugation	VSVG-retrovirus	82	220	NM	96	NM	90 min	Not tested VSVG can induce cell-cell fusion	In vitro	NM	50000g, 90 min, 4°C	[28]
Ultracentrifugation	VSVG, amphotropic and rabies-G lentivirus	30	86-300	NM	96-116	NM	~3hrs	NM	In vitro	NM	25000 rpm (Beckman SW28), 105 min, 15°C	[45]
Ultracentrifugation	RD114-retrovirus	~240	~ 300-480	NM (high % transduction and engraftment)	50-70		90 min	NM	In vitro/ ex vivo (reimplant)	Purified from agent that prevents engraftment	100000g 90 min	[30]

Table 1.1 Methods for concentration of virus stocks (continue)

Concentration Method	Virus	Starting volume (mL)	Fold volume reduction	Fold increase in transduction	Recovery by titer (%)	Recovery of virus particles	Time required	Cytotoxicity	Tested	Purification from inhibitors/other?	Details	Ref
COMPLEXATION OF VIRUSES [d]												
Paramagnetic particles	Retrovirus	5	125	NM	2-3360 [g]	NM	~ 2 hrs	NM	In vitro	NM	Incubate 2 hrs, 4°C, constant motion.	[31]
Complexation with Pansorbin	Retrovirus	45-50	50-250	NM	78-792	NM	1-2 hrs	Speculate toxicity in vivo	In vitro	NM	Incubate 1-2 hrs, 4°C. Centrifuge 2600g, 10 min, 4°C	[24]
Complexation with Poly-L-lysine	VSVG-lentivirus	10-3000	20-500	NM	26-37.5	NM	~2.5 hrs	No significant toxicity in vivo	In vitro/ in vivo	NM	Incubate 30 min, 4°C. Centrifuge 1000g, 2 hrs, 4°C Better virus recovery and titer than ultracentrifugation	[39]
Co-precipitation wih calcium phosphate	Amphotropic retrovirus and lentivirus	10-500	10-100	NM	46-200	NM	6-7 hrs	NM	In vitro	NM	Virus produced in serum-free medium. Incubate 37°C, 30 min, Centrifuge ,2060g, 4 min. Resuspend in EDTA, Dialyze for 6 hrs at 4°C.	[20]

a: High pressure compromises viral bioactivity, membrane can be clogged, high molecular weight inhibitors can be co-concentrated
b: ≤ 2000g. May co-concentrate inhibitors.
c: > 2000g. Limited to viruses with selected characteristics.
d: Flocculation of chemical conjugation of viruses with agents that permit their rapid recovery by short, low speed centrifugation or via a magnetic field. Virus complexes can sediment ex-vivo, thereby overcoming virus diffusion limitations.
e: Plateau develops for virus concentration dose response. Titer does not predict transduction
f: Purification by sucrose banding
g: Increase in percentage transduction. Increasing viral titers to $>10^8$ failed to further enhance gene transfer.
h: Fold enhancement depends on producer cell and chemical conjugation of paramagnetic particles and viruses.
NM: not mentioned

understood, but it has been shown that glycosaminoglycans decrease the rate of binding of virus particles to cells [40, 43, 44, 48]. Binding appears to be mediated by non-specific interactions between the surface of the virus and glycosaminoglycans expressed on the surface of the cells [48], or by interactions between glycosaminoglycans on the surface of the viruses and binding sites on fibronectin [26]. Hence, free glycosaminoglycans are likely to compete with cell-associated glycosaminoglycans for virus binding [44, 48] or with virus-associated glycosaminoglycans for binding to the cells through fibronectin [26].

On the other hand, soluble (free) viral envelope proteins are present in retrovirus stocks and inhibit transduction [42, 49-52]. During virus production, some of the envelope proteins are not incorporated into virus particles, but shed from packaging cells [51, 52]. Also, it is possible that free envelope sheds from virus particles. Early studies suggested that the shedding of envelope proteins was a common phenomenon of retroviruses and lentiviruses [53-55], but more recent quantitative studies of the protein content of retroviruses and lentiviruses have demonstrated that envelope proteins are more stably associated with virus particles than previously thought and that lentiviruses shed few, if any, envelope proteins [56, 57]. Soluble viral envelope act as a competitive inhibitor of virus-associated envelopes for binding to cell receptors and thus interfere with the fusion step of transduction [42, 58].

In order to purify virus stocks from inhibitors, various techniques have been developed. Some of the concentration procedures we mentioned previously can achieve purification of stocks from serum proteins and certain inhibitors [31, 45]. However, these techniques may also co-concentrate inhibitors with virus particles [59, 60], making it necessary to conduct multi-step processing of virus stocks. Procedures aimed exclusively at purifying virus stocks can be applied to concentrated stocks, and include

anion exchange chromatography, hydroxyapatite chromatography and sucrose gradient centrifugation [27, 32, 35, 36, 61, 62].

Most of the purification and concentration methods mentioned are time consuming, require elaborate pieces of equipment and result in the loss of a significant fraction of the viruses originally present in stock. As a result, multi step processes cause substantial loss of virus particles and require long processing times [32].

Inefficient interactions between viral envelope proteins and cellular receptors

The specific interaction between viral envelope glycoproteins and receptors on the surface of target cells is another major determinant of the efficiency of transduction. After viruses bind non-specifically to the surface of the cells [15-17, 48, 63], viral envelope glycoproteins interact with cell receptors, which mediates fusion and entry of the viral core. Envelope proteins consist of two subunits: the extracellular domain SU (gp70) and the transmembrane domain TM (Prp15E) [64, 65]. These two subunits result from cleavage of a single precursor protein, $gPr80^{env}$, and remain associated after cleavage. The SU subunit mediates binding of the virus particle to cell receptors, while SU and TM act in conjunction to mediate fusion and entry of the virus inside the target cell [66, 67].

The choice of the envelope protein is a major determinant of gene transfer, as the specific interaction with cell receptors determines the range of cells that a virus can or cannot transduce (the tropism of the virus). In order to target specific cells in gene therapy protocols, the wild type envelope of a virus can be replaced with one from another virus to form a pseudotyped virus (a virus composed of proteins from more than one virus) [68, 69]. Commonly used envelope proteins include the amphotropic, ecotropic, 10A1, gibbon ape leukemia virus (GALV) and the vesicular stomatitis virus G (VSV-G) protein. During virus production, these proteins are transported to the surface

or to intracellular compartments of packaging cells, where they are incorporated into virus particles. This incorporation has been thought to take place at the plasma membrane, during budding of the virus [70, 71]. However, recent evidence suggests that colocalization of the envelope and other viral proteins occurs in the cytoplasm [72, 73]

In addition to tropism, the choice of the envelope determines the efficiency of gene transfer [33, 74]. In fact, low expression of receptors has been shown to correlate with poor transduction [51, 75-80]. In some cases, transduction has been enhanced by inducing overexpression of receptors through manipulation of the cell culture medium. For example, the amphotropic receptor (Pit-2) and the gibbon ape leukemia virus receptor (Pit-1) are upregulated by incubation of the cells in phosphate depleted medium or in the presence of phorbol 12-myristate 13-acetate (PMA) [76, 81-83]. In hematopoietic stem cells, these receptors can be upregulated by incubation of the cells with cytokines [77, 80, 84]. Alternatively, it is possible to overexpress the receptor by transfecting the cells with the gene encoding for it [77, 83]. Slight upregulation of receptors that are normally expressed at low levels has been shown to translate in major increases in gene transfer [77]. However, this type of manipulation of target cells is not always suitable for gene therapy applications. First, most of these procedures require *ex vivo* treatment of the cells. Second, these treatments can potentially result in an altered phenotype of the target cells after reimplantation into the body.

The efficiency of envelope-receptor interactions not only depends on the number of cell receptors, but can also be altered by varying the number of envelope glycoproteins per virus. The number of envelope proteins on the surface of a virus depends on the level of expression of these proteins by packaging cells [42, 85]. It also depends on the recognition of the envelope by the viral core during assembly of the virus particles [73, 86, 87]. Envelopes from a single virus form oligomers and act cooperatively to achieve efficient fusion [65, 87, 88]. Higher concentrations of virus-

associated envelope proteins have been shown to correlate with higher rates of transduction [85]. Although the exact mechanism of fusion is not clear, it has been proposed that binding of multiple envelope proteins to multiple receptors creates a tighter binding that leads to faster fusion [85, 89]. In agreement with this idea, increasing the fluidity of the plasma membrane by increasing the temperature or by incubating cells with a detergent, has been shown to increase viral infectivity [90]. This is probably due to the fact that a more fluid membrane permits rapid mobilization of receptors to the site of virus binding, thereby providing multiple binding sites for multiple envelope proteins of a virus particle.

While several studies have shown that increasing the number of envelopes per virus increases transduction , other studies suggest that there is an optimum level of envelope expression that maximizes transduction [42]. In fact, an increase in the expression of viral envelope may correlate with an increase in shedding of free envelope from packaging cells, and thereby with an increase in inhibitory activity in the virus stocks. Also, it is possible that an excess of viral envelopes associate with non-functional virus particles and compete with envelopes from functional viruses for binding to cell receptors [42]. A better understanding of how adjusting the level of envelope expression can be used to maximize or control the efficiency of transduction is required to develop safe and efficient viral vectors.

1.4 Thesis objectives

The objectives of this thesis were to enhance the efficiency of transduction, and to improve the reproducibility and predictability of gene transfer using recombinant retroviruses as gene delivery vectors. To accomplish these objectives, we pursued the following aims:

1) Develop and characterize a method to overcome the major extracellular rate-limiting steps of gene transfer.
2) Determine the extent to which increasing the rate of transport of viruses to cells enhances gene transfer.
3) Determine the extent to which the composition of the medium in virus stocks affects the efficiency of transduction, and evaluate the effect that purifying viruses from inhibitors in the conditioned medium has on gene transfer.
4) Examine of the relationship between the level of gene transfer and the number of envelope proteins that are incorporated per retrovirus particle when high doses of purified virus stocks are used to transduce cells

1.5 Organization of the thesis

In chapter one, we briefly introduce gene therapy and the use of recombinant retroviruses as gene delivery vectors. A major emphasis is placed on limitations of retroviral-mediated gene transfer.

In chapter two, we develop a method to substantially increase the efficiency of retroviral-mediated gene transfer. The method consists of inducing aggregation (flocculation) of virus particles with charged polymers, which results in the formation of high molecular weight virus-polymer complexes. We evaluate the possibility of using these complexes to rapidly concentrate and purify virus stocks. Also, we evaluate the utility of this method to improve gene transfer to different target cells with various pseudotyped retroviruses.

In chapter three, we examine how aggregation of viruses into polyelectrolyte complexes affects the rate of binding of viruses to cells. We find that increasing the concentration of polymers in these complexes increases the size of the virus-polymer complexes and the rate of transport of viruses, while not interfering with post-binding

steps of transduction. Based on these findings, we examine the relationship between virus binding and transduction efficiency.

In chapter four, we examine the possibility of flocculating viruses with different combinations of charged polymers. Also, we evaluate the advantages and disadvantages of using a single species of cationic polymer versus using two species of oppositely charged polymers to induce flocculation of viruses and to enhance transduction.

In chapter five, we use polyelectrolyte complexes to purify virus stocks from inhibitors of transduction that are present in virus stocks. We examine the nature of the main inhibitor in our system, which appears to be free viral envelope protein. Also, we evaluate the effect of purification on the efficiency of transduction.

In chapter six, we construct a panel of several clonal virus packaging cell lines, which differ only in the amount of envelope protein that they express. We purify virus stocks produced by these cell lines from free envelope, and we examine how varying the concentration of virus-associated envelope protein affects transduction.

In chapter seven, we summarize our major conclusions and present some suggestions for future work.

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CHAPTER 2

COMPLEXATION OF RETROVIRUSES WITH CATIONIC AND ANIONIC POLYMERS INCREASES THE EFFICIENCY OF GENE TRANSFER*

2.1 Abstract

The combined addition of equal weight concentrations (80 μg/ml) of the cationic polymer Polybrene and the anionic polymer chondroitin sulfate C to retrovirus stocks resulted in the formation of high molecular weight retrovirus-polymer complexes that could be pelleted by low speed centrifugation. The pelleted complexes contained more than 80% of the virus particles, but less than 0.3% of the proteins that were originally present in the virus stock. Surprisingly, the viruses in the complexes remained bioactive and could be used to transduce cells. The titer of the pelleted virus, when resuspended in cell culture medium to the starting volume, was 3-fold greater than the original virus stock. The selectivity (cfu/mg protein) of the process with respect to virus activity was more than 1000-fold. When the pelleted virus/polymer complex was resuspended in one-eighth of the original volume and used to transduce NIH 3T3 murine fibroblasts, gene transfer was increased 10 to 20-fold over the original unconcentrated retrovirus stock.

2.2 Introduction

Recombinant retroviruses are frequently used for experimental and clinical gene transfer because of their ability to permanently modify the genome of cells, their ability to transfer genes to a wide variety of primary human cell types, and the relative ease of

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their construction and production. Despite these advantages, recombinant retroviruses have had limited success in human gene therapy clinical trials, in part because they fail to transfer enough genes to achieve a required biological effect [1-5].

Retrovirus infection requires the successful completion of a complex series of steps that begins with the transport and binding of the virus particle to the surface of the cell. Binding has been thought occur through specific interactions between the envelope proteins that protrude from the surface of the virus particle and the receptors for the virus that are expressed on the surface of the target cells [6]. However, recent studies have shown that the initial step of virus binding does not require specific envelope-receptor interactions and that the efficiency of retrovirus infection appears to correlate well with the efficiency of an initial, receptor-independent, binding event [7, 8]. These observations suggest that it may be possible to substantially increase the efficiency of gene transfer by improving the rate at which retroviruses complete this initial, receptor-independent, binding step. In this context, charged polymers, which are thought to act on an early receptor-independent step of infection, have been shown to alter the efficiency of retrovirus infection [9, 10]. Cationic polymers, such as poly-l-lysine, Polybrene, and protamine enhance infection by as much as ten-fold, possibly by reducing the electrostatic repulsion between retroviruses and cells. Likewise, anionic polymers such as dextran sulfate and heparin reduce the transduction efficiency of retroviruses and are thought to decrease binding by increasing the electrostatic repulsion between viruses and cells [10].

Polyanionic polymers that are naturally secreted by cells, such as chondroitin sulfate proteoglycans and glycosaminoglycans, have been shown to inhibit retrovirus transduction [11-13]. Interestingly, the efficiency of infection is slightly enhanced, rather than inhibited, when low doses of the glycosaminoglycan chondroitin sulfate C (CSC) are used in conjunction with equal weight concentrations of Polybrene (PB) [14]. These

results suggested that it might be possible to further enhance the efficiency of gene transfer if higher doses of these polymers were used and if the mechanism of enhancement was understood. Therefore, in this study we tested the hypothesis that inclusion of high concentrations of CSC and PB would substantially enhance the efficiency of retrovirus-mediated gene transfer.

2.3 Materials and methods

Chemicals and antibodies. Nonidet P-40, o-nitrophenyl-β-D-galactopyranoside (ONPG), glutaraldehyde, rhodamine B, 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT), 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (Polybrene), *o*-phenylenediamine (OPD), and chondroitin sulfate C (CSC) (shark cartilage, 59 kDa) were purchased from Sigma Chemical Co., St Louis, MO. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was purchased from Denville Scientific Inc., Metuchen, NJ. BLOTTO® Blocker in TBS was from Pierce, Rockford, IL. Mouse anti-p30 antibodies were purified from the supernatant harvested from CRL-1912 hybridoma cell line (ATCC, Rockville, MD) following standard protocols [15]. The goat polyclonal anti-p30 antibody (78S221) was from Quality Biotech, Camden, NJ. The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories, South San Francisco, CA. The PDGF-AB ELISA Kit was purchased from R&D Systems, Minneapolis, MN.

Cell culture. NIH 3T3 fibroblasts, the amphotropic packaging cell line ψ-CRIP [16] and the ecotropic packaging cell line Cre Bag 2 (ATCC, CRL-1858) were cultured in Dulbecco's modified Eagle's medium with 10% bovine calf serum (HyClone Labs Inc., Logan, UT) containing 100 U/mL of penicillin and 100 μg/mL of streptomycin (Gibco BRL). CHO-K1 Chinese hamster ovary cells were cultured in Ham's F12K medium with

10% fetal bovine serum (HyClone Labs, Inc., Logan UT). 293T/17 human embryonic kidney (ATCC) and GP2-293 cells were cultured in DMEM, 10% fetal bovine serum (Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 110 μg/mL of sodium pyruvate (DMEM/FBS).

Production of retroviruses. To generate retrovirus stocks, virus-producing cells were grown to confluence in T175 tissue culture flasks, and then incubated for 24 h with 35 mL of DMEM/BCS. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use. We produced various pseudotyped retrovirus vectors encoding for GFP by transient transfection of GP2-293 cells that were plated the day before in 10-cm tissue culture dishes ($6x10^6$ cells/dish), and cultured in 10 mL of DMEM that contained no antibiotics. The next day, cells were transfected with the MSCV-GFP vector (kind gift of Catherine Verfaillie) and with FB4070ASALF (for amphotropic virus), FBMO-SALF (for ecotropic virus), pMD.G (for VSV-G virus), pIK.MCV.10A1.env.uTd (for 10A1 virus), pCIGASENV (for GALV virus) or no other plasmid (for bald virus). Eight micrograms of each plasmid were diluted into a total of 1.5 mL of DMEM, then mixed with a solution of 1.5 mL of DMEM and 40 μL of Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA), then added to the cells. The medium was replaced 6 hours later and 12 hours later with 10 mL of DMEM/FBS. Twenty four hours later the virus-laden cell culture supernatant was harvested, filter sterilized (0.45-μm) and frozen (-80°C) for later use.

MTT assay for cell number. To test the effect of the CSC and Polybrene (PB) on the growth rate, NIH 3T3 murine fibroblasts were plated in parallel 96 well plates at 5000 cells per well, then incubated 24 h at 37°C. The cell culture medium was then replaced with medium that contained from 0 μg/ml to 640 μg/ml of CSC, and 0 μg/ml to 640 μg/ml of PB, and the cells incubated at 37°C. Control cells were grown in the

presence of fresh medium that did not contain any CSC or PB. To measure the number of viable cells, two days later 10 μl of an MTT stock (10 mg of MTT per ml in PBS, fitler sterilized) was added to each well. After incubation of the plate at 37°C for 4 h, the cells containing dark blue formazan precipitate were solubilized by addition of 150 μl of 10% sodium dodecyl sulfate (SDS) and incubation overnight at 37°C. The absorbance at 570 nm was read, and nonspecific background at 650 nm subtracted.

Transduction efficiency assay. To test the effect of CSC and PB on transduction efficiency, the day before transduction five thousand cells were plated in 100 μl of medium per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, MA). The next day (19 to 25 h later), virus stocks were brought to a concentration of CSC ranging from 0 μg /ml to 20 μg/ml, incubated for 10 min at 37°C, then brought to 8 μg/ml PB and incubated an additional 10 min at 37°C. Alternatively, virus stocks were brought to a concentration of CSC ranging from 0 μg/ml to 200 μg/ml, incubated for 10 min at 37°C, then brought to 80 μg/ml PB and incubated an additional 10 min at 37°C. The medium was removed, and the virus-polymer mixtures added to each well (final volume, 100 μl per well). Two days after transduction, the medium was removed, and the cells were washed once with 100 μl of phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$. After removal of the wash solution, 50 μl of lysis buffer was added (PBS with 1 mM $MgCl_2$ and 0.5% nonidet P-40) to each well, and the plate was incubated at 37°C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG warmed to 37°C was added to each well, and the plate was incubated at 37°C for another 15 min [17]. The reactions were halted by the addition of 20 μl of stop buffer (1 M Na_2CO_3) [18]. The plate was brought to room temperature, and the optical density at 420 nm (OD_{420}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA); non-specific background at 650 nm was subtracted. Values

for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Virus precipitation and resuspension. To determine the concentration of polymers needed to form a complex with retrovirus, stocks were brought to a range of concentrations of CSC (0 to 160 μg/ml) and to an equal weight concentration of PB, then incubated for 20 minutes at 37°C. In later experiments, the retrovirus-polymer complex was formed by bringing virus stocks to 80 μg/ml of CSC and 80 μg/ml of PB. Once the polymers had been added, a portion of the virus solution was removed for analysis, and the remainder centrifuged at 10000 g in a 1.5 mL microcentrifuge tube in an Eppendorf centrifuge for 5 minutes at room temperature. The supernatant was removed for analysis, and the visible pellet resuspended in PBS or cell culture medium to its original volume or to one-eighth its original volume. All samples were then analyzed for virus titer, transduction efficiency, concentration of virus proteins, and/or total protein concentration. An additional eight micrograms of PB was added per ml of sample prior to their use in the transduction efficiency assay.

Detection and quantitation of MoMuLV p30 capsid protein by ELISA. To determine the concentration of virus capsid protein (p30) in virus stocks, we used an ELISA. ELISA plates (Nunc immuno Maxisorp 96-well plates, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to block non-specific binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 was sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer, and incubated for 1 h at 37°C. The horseradish

peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed for 5 min using hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Diluted virus titer assay. Serial dilutions of *LacZ* virus stock were made in DMEM with 10% bovine calf serum and PB (8 μg/ml). 2 mL were used to transduce cells in 6 well plates (Costar Corp., Cambridge, MA) seeded at 1.5 x 10^5 NIH 3T3 cells/well the previous day. The transduced cells were incubated for two days at 37°C until confluent, fixed and stained for *lacZ* activity with XGAL, colonies of *lacZ*+ cells counted and the titer (cfu/ml) calculated as previously described [19].

Protein concentration. Protein concentrations were determined with the Coomassie Plus-200 Protein Assay Reagent in accordance with the manufacturer's instructions (Pierce, Rockford, IL). Bovine serum albumin was used as the standard.

Virus tropism experiments. To determine if retrovirus-polymer mixtures were able to transduce cells that did not express the receptor for the retrovirus, amphotropic and ecotropic retrovirus-polymer complexes were formed and pelleted with 80 μg/ml of CSC and 80 μg/ml of PB as described above. The pellets were resuspended in cell culture medium that was appropriate for the target cell type, then used to transduce NIH 3T3 murine fibroblasts, 293T/17 cells, and CHO-K1 Chinese hamster ovary cells.

2.4 Results

In previous research, small but statistically significant increases in the efficiency of retrovirus transduction were observed when virus stocks were brought to equal weight concentrations of the cationic polymer Polybrene (PB) and the anionic polymer chondroitin sulfate C (CSC) prior to transduction [14]. These results prompted us to investigate whether substantial increases in retrovirus-mediated gene transfer could be achieved using these polymers.

Our first experiments showed that gene transfer efficiency increased in proportion to the concentration of polymers, which suggested that further increases in the concentration of polymers might result in even higher gene transfer efficiencies. To determine if high concentrations of polymers were cytotoxic, we incubated NIH 3T3 fibroblasts with a range of concentrations of Polybrene (PB) only, chondroitin sulfate C (CSC) only, or a mixture of both PB and CSC for two days, then measured the final cell number with the MTT assay (Figure 2.1). We found that PB, but not CSC, was cytotoxic at high concentrations. However, mixtures of equal weight concentrations of PB and CSC were not cytotoxic, suggesting that CSC mitigates the cytotoxic effects of PB.

Since high concentrations of PB and CSC mixtures were not cytotoxic, we decided to investigate if higher doses of polymers could be used, and to observe how transduction efficiency would vary with changes in concentration and with changes in the the ratio of the two polymers. Virus stocks were brought to low doses of PB (8 μg/ml) or to high doses of PB (80 μg/ml), and then to range of concentrations of CSC. These stocks were used to transduce cells. As a control, cells were transduced with virus that contained only 8 μg/ml PB. Regardless of the concentration of PB used, the transduction efficiency increased, reached a peak, then decreased with increasing concentrations of CSC (Figure 2.2). Gene transfer was enhanced more than three-fold when high doses of PB

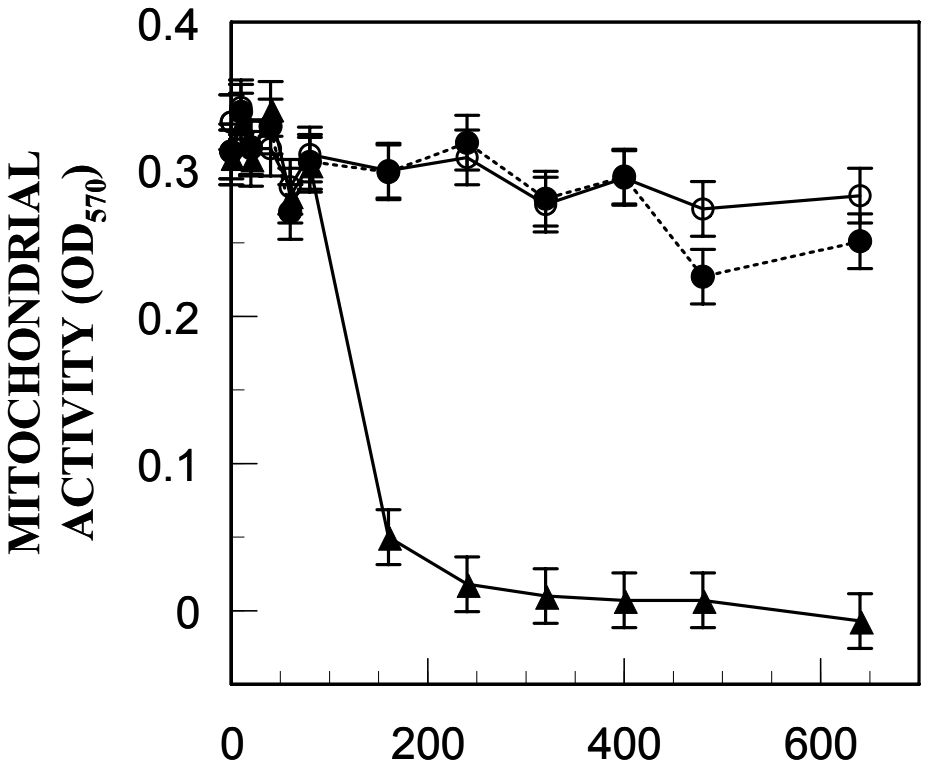

Figure 2.1 Mixtures of PB and CSC are not cytotoxic. NIH 3T3 cells were grown in the presence of various concentrations of CSC (●), PB (▲), or equal weight concentrations CSC and PB (O). After two days of growth, mitochondrial activity was determined using the MTT assay. Each point shows the mean ± standard deviation of three replicates.

(80 μg/ml) and CSC were used, and less than 2-fold when low doses of PB (8 μg/ml) and CSC were used. of PB (80 μg/ml) and CSC were used, and less than 2-fold when low doses of PB (8 μg/ml) and CSC were used. As expected due to its cytotoxicity, gene transfer was negligible when high doses of PB (80 μg/ml) were used in the absence of CSC. Interestingly, regardless of the absolute concentration of the polymers, the highest transduction efficiencies were observed when the weight concentrations of CSC and PB were about equal. Thus, for the remaining studies, we used equal weight concentrations of CSC and PB.

In the course of these experiments we noted the presence of granular precipitates on the surface of the cells that had been exposed to 80 μg/ml PB and 80 μg/ml CSC. This suggested that PB, CSC, and perhaps viruses, had formed complexes that sedimented onto the cells. To determine if retrovirus had incorporated into polymer complexes, we brought virus stocks to equal concentrations of CSC and PB ranging from 0 to 160 μg/ml each, then centrifuged the stocks at 10000g for 5 minutes. We obtained a visible pellet that we resuspended to its original volume in fresh medium. We measured the concentration of virus capsid protein (p30) in the pellet by ELISA. Levels of p30 in the pellet increased with increasing concentrations of polymers until, at 80 μg/ml of each polymer, more than 85% of p30 was found in the pellet (Figure 2.3). At greater than 80 μg/ml, no further increases in the amount of p30 in the pellet were observed.

To determine if incorporation of viruses into polymer complexes was dependent on the envelope protein, we brought stocks of viruses not displaying an envelope protein (bald viruses) and stocks of viruses pseudotyped with the amphotropic, ecotropic, VSVG, 10A1 or GALV envelope protein, to 80 μg/mL of CSC and to 80 μg/mL of PB. We pelleted these stocks and resuspended the pellets to their original volume in fresh

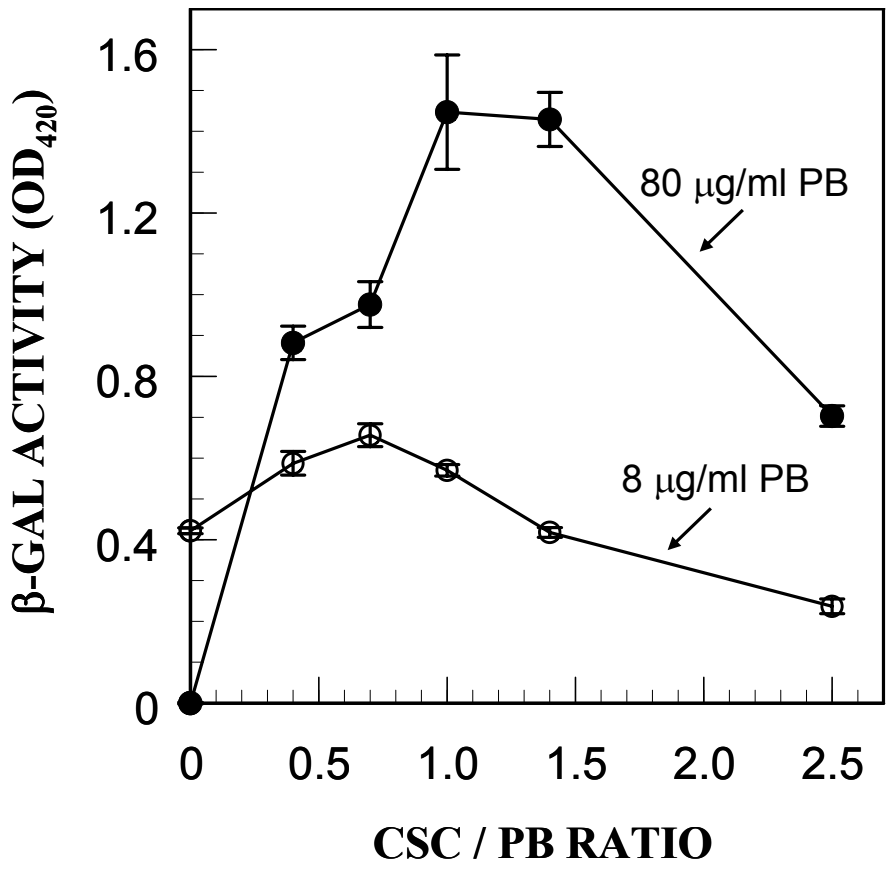

Figure 2.2 Mixtures of CSC and PB enhance gene transfer. NIH 3T3 cells (5000 per well) were plated in a 96 well dish and the next day transduced with 100 μl of medium containing amphotropic *lacZ* virus, 8 μg/ml PB (O) or 80 μg/ml PB (●), and a weight concentration of CSC ranging from 0 to 2.5-fold that of PB. After two days the cells were assayed for β-galactosidase activity. Each point shows the mean ± standard deviation of three replicates.

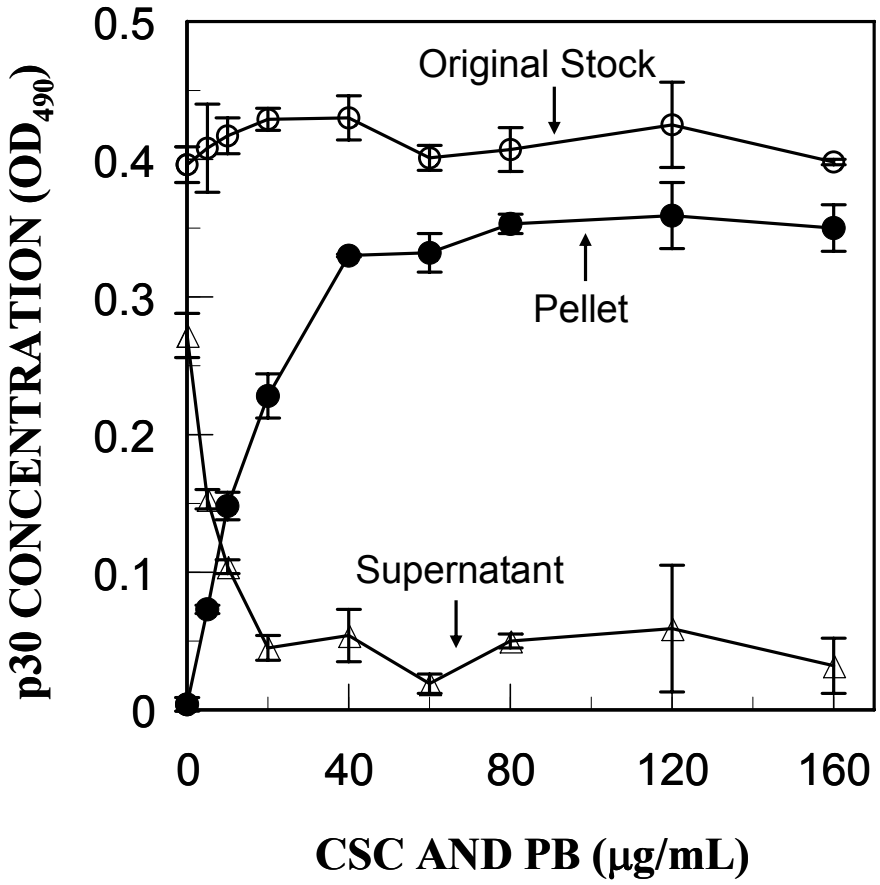

Figure 2.3 Retrovirus particles form complexes with PB and CSC. A stock of amphotropic *LacZ* virus was brought to a range of equal weight concentrations of CSC and of PB, incubated at 37°C, then pelleted by centrifugation. The pellet was resuspended to the original volume in fresh medium. The concentration of virus in the supernatant (△), resuspended pellet (●), and original stock with added PB and CSC prior centrifugation (O), was determined with the p30 ELISA. Each point shows the mean ± standard deviation of three replicates.

medium. We found that viruses were incorporated into the complexes with equal efficiency, regardless of which envelope protein was used to pseudotype them, and even when bald viruses were used (Figure 2.4). To determine if the viruses in the polymer complex were still active, we brought a stock of amphotropic viruses to 80 μg/ml CSC and 80 μg/ml PB, centrifuged and resuspended the resulting virus-polymer pellet to its original volume in fresh cell culture medium, and then used it to transduce NIH 3T3 cells. The titer of virus that had been pelleted and resuspended to its original volume, was three-fold higher than the titer of the original virus stock (Table 2.1). Similar increases in gene transfer as measured by the transduction efficiency assay were observed when the pelleted and resuspended virus was applied directly to cells in undiluted form. Interestingly, increases in titer were not observed in virus stocks that were brought to 80 μg/ml of CSC and 80 μg/ml PB but not pelleted (Table 2.1). The titer of the supernatant that remained after centrifugation of the virus-polymer complex was variable but low (9 ± 7% of the original virus titer, n=5), as expected, indicating that most of the virus had been pelleted.

To determine the selectivity of the complexation process, we measured the total protein concentration in the supernatant and the pellet as well as in the original virus stock before and after addition of the polymers (Table 2.1). The protein concentration in the pellet was less than 0.3 percent of the protein concentration in the virus stock prior to centrifugation, whereas the protein concentration in the supernatant was indistinguishable from the original stock.

Since the use of high concentrations of polymers was not cytotoxic and pelleting of the complexes did not inactivate the viruses, we decided to test if concentrated virus stocks, generated by pelleting and resuspension of the virus-polymer complex in reduced volumes of cell culture medium, could be used to transduce cells. We

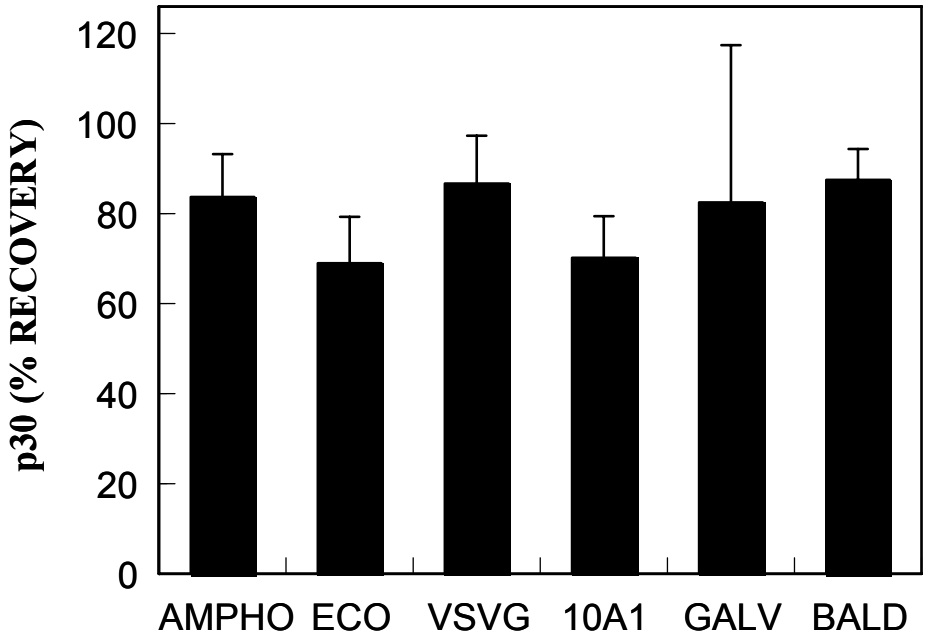

Figure 2.4 Retroviruses are incorporated into complexes regardless of the pseudotype. Viruses pseudotyped with the amphotropic (A), ecotropic (E), VSVG, 10A1, GALV, or no (BALD) envelope protein, were brought to 80 μg/mL CSC and PB, pelleted, and then resuspended to their original volume. The amount of virus in the pellet was determined by p30 ELISA and is expressed as a percent of the virus from the original stock. Each point represents the mean ± standard deviation of triplicate samples.

Table 2.1 Purification of retrovirus by formation of a virus-polymer complex[a]

Sample	Total Protein (μg/ml)	Virus capsid protein p30 (OD490)	Virus Titer[b] (cfu/ml)	Transduction Efficiency (OD 420)
Before Pelleting	6900 ± 800	0.37 ± 0.04	37,000 ± 7200	1.3 ± 0.04
After Pelleting	20 ± 4.0	0.29 ± 0.04	110,000 ± 30,000	2.4 ± 0.30
% Recovery	0.29 ± 0.07	78 ± 14	300 ± 100	180 ± 24

[a]*LacZ* virus was brought to 80 μg/ml CSC, incubated for 10 min at 37°C, brought to 80 μg/ml of PB, then incubated for an additional 10 min. A portion of this virus-polymer suspension was saved for later analysis (Before Pelleting); the remainder was centrifuged at 10,000 rpm in a 1.5 ml microcentrifuge tube for 5 min at room temperature. The supernatant was removed and the pelleted virus-polymer complex was resuspended to the original volume in PBS (After Pelleting). The samples were then analyzed for total protein content, concentration of p30, virus titer and transduction efficiency as described in the materials and methods. Values are means ± SD for at least three replicates.

[b]The titer of the virus stock used in these experiments was 37,000 ± 12,000 cfu/ml. Similar results were observed when higher titer (greater than 10^5 cfu/ml) virus stocks were used.

centrifuged a virus stock that contained 80 μg/ml of CSC and 80 μg/ml of PB, then resuspended the pellet in one-eighth of the original volume with cell culture medium. We used the concentrated virus to transduce NIH 3T3 fibroblasts. The concentrated virus-polymer complex transduced NIH 3T3 fibroblasts more than 10-fold more efficiently than the unconcentrated virus-polymer mixture, and more than 20-fold more efficiently than the original virus stock that contained no polymers (Figure 2.5).

To determine if the tropism of the virus was altered by complexation with the polymers, we brought stocks of ecotropic and amphotropic retroviruses to 80 μg/ml of CSC and PB. We pelleted the stocks, resuspended the pellets in fresh culture medium, and used them to transduce NIH 3T3 fibroblasts, 293T/17 cells, and CHO-K1 cells. Amphotropic and ecotropic viruses were only able to transduce cells that expressed the amphotropic and ecotropic receptor, respectively, regardless of whether or not they had been complexed with CSC and PB (Figure 2.6).

Taken together, our results indicated that complexation of viruses with charged polymers permits to efficiently and easily concentrate virus stocks within less than 30 min (including 20 min of incubation of viruses with polymers to allow formation of the complexes and 5 min for pelleting of the complexes). In a effort to minimize the time required to conduct this procedure, we varied the incubation time of the virus stock with the polymers between 0 and 20 min (while maintaining the centrifugation time at 5 min) and the centrifugation time between 0 and 5 min (while maintaining the incubation time at 20 min), then quantified the recovery of p30 in the pellet. Incubation of the virus stock with PB and CSC for 5 minutes and centrifugation of the stock for 3 minutes resulted in optimal recovery of p30 (Figure 2.7). Also, a 5-min incubation followed by a 3-min centrifugation resulted in levels of gene transfer similar to those obtained previously

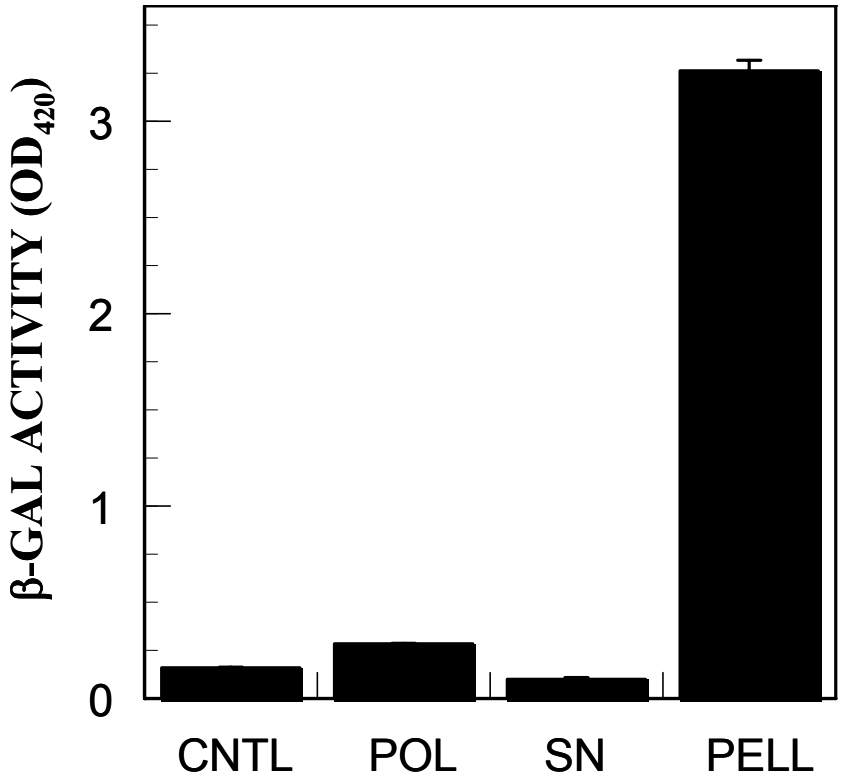

Figure 2.5 Retrovirus-polymer complex can transduce cells. Amphotropic *LacZ* virus was brought to 80 μg/ml CSC and PB and the resulting virus-polymer complex pelleted, then resuspended, to one-eighth of the original volume. NIH 3T3 cells were transduced with: control starting virus with no added polymers (CNTL), the starting virus with added polymers prior to centrifugation (POL), the supernatant (SN), or the virus-polymer complex resuspended to 1/8 the original volume (PELL). All samples were brought to a final concentration of 8 μg/ml of PB prior to transduction. Transduction was measured using the transduction efficiency assay that measures the level of β-galactosidase activity in the transduced cells. Each point shows the mean ± standard deviation of three replicates.

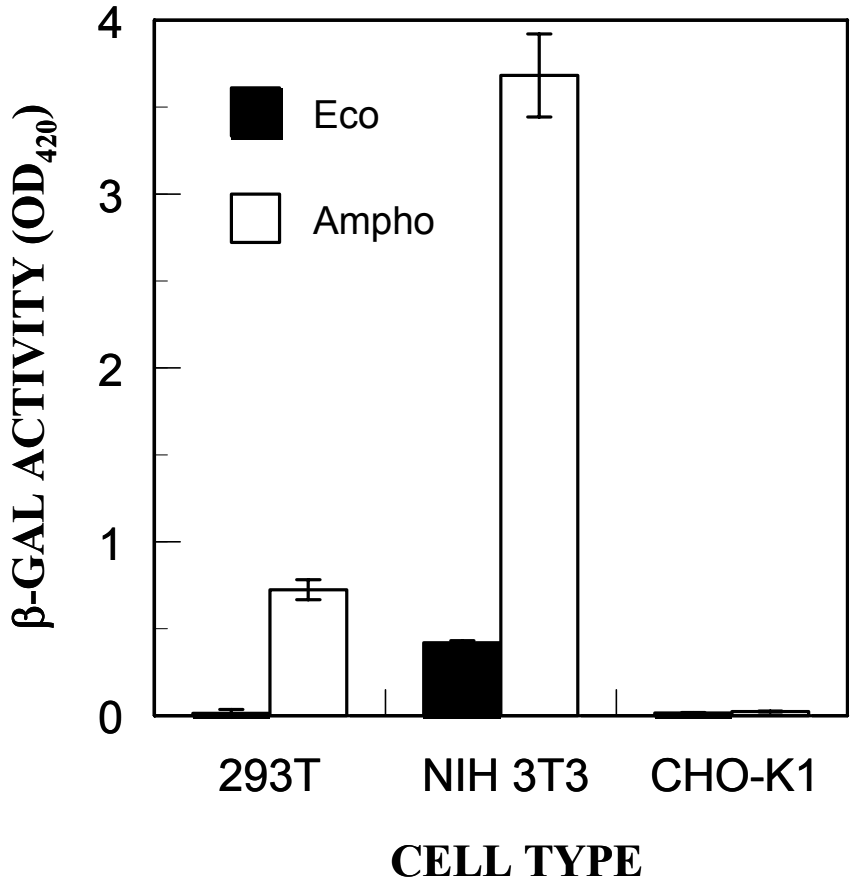

Figure 2.6 Virus tropism is not altered by the formation of a virus-polymer complex. Ecotropic (black bars) and amphotropic (white bars) *lacZ* viruses were brought to 80 μg/ml CSC and PB and the resulting virus-polymer complex pelleted, then resuspended, to their original volume in cell culture medium that was appropriate for the intended target cell type. Resuspended pellets were used to transduce human embryonic kidney cells (293T/17)(permissive for amphotropic), murine fibroblasts (3T3)(permissive for ecotropic and amphotropic), or Chinese hamster ovary cells (CHO-K1)(nonpermissive for ecoptropic and amphotropic). Transduction was measured using the transduction efficiency assay that measures the level of β-galactosidase activity in the transduced cells. Each point shows the mean ± standard deviation of three replicates.

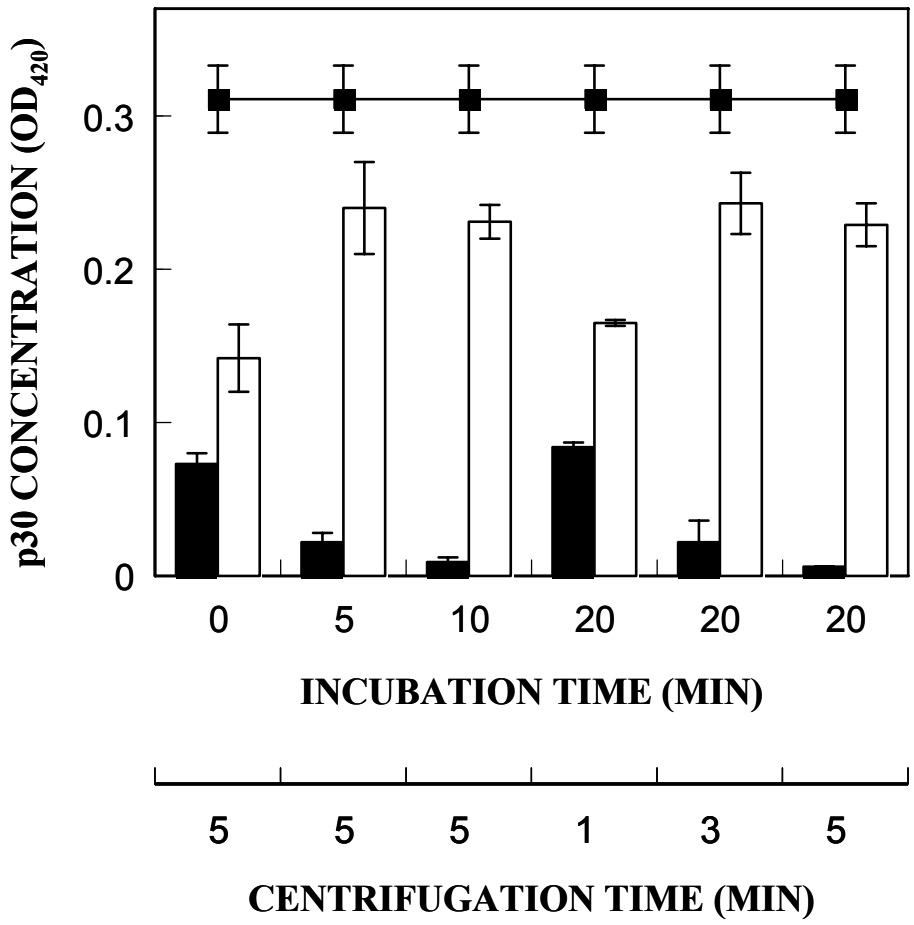

Figure 2.7 Optimization of the incubation and centrifugation times for pelleting of viruses with polymers. Stocks of amphotropic virus were brought to 80 μg/mL of PB and 80 μg/mL of CSC, incubated for 0, 5, 10 or 20 min at 37°C, then centrifuged at 10000g for 5 min. Also, virus stocks were incubated for 20 min at 37°C, then centrifuged at 10000g for 1, 3 or 5 min. The supernatants were collected and the pellets resuspended to their original volume. The concentration of p30 in the original virus stock (line above the bars), the supernatants (white bars) and the pellets (black bars) was measured by ELISA. Each point represents the mean ± standard deviation of triplicate samples.

(data not shown). These results indicated that concentration of virus stocks is possible in less than 10 min.

2.5 Discussion

Addition of high and equal weight concentrations of the cationic polymer PB and the anionic polymer CSC resulted in the formation of visible virus-polymer complexes that incorporated 80% of the virus particles, but fewer than 0.3% of the proteins that were originally present in the virus stock. PB and CSC mixtures were not cytotoxic even when concentrations as high as 640 μg/ml of each polymer were used. When the virus-polymer complexes were formed by the addition of 80 μg/ml of CSC and 80 μg/ml of PB, the efficiency of gene transfer increased by more than 3-fold. Centrifugation of these complexes resulted in the formation of a visible pellet. Resuspension of the pellet to one eighth the original volume resulted in a 10 to 20-fold increase in gene transfer to NIH 3T3 cells.

The mechanism by which the oppositely charged polymers and the negatively charged virus form a complex is not known, but our results indicate that high concentrations of both polymers must be added to get significant precipitation of the virus. In addition, our results demonstrate that the formation of the complexes is not the result of specific interactions with the virus envelope protein since non-enveloped viruses and various pseudotyped viruses formed complexes equally well with the polymers. Also, in this study we did not examine the mechanism by which the complex enhances the efficiency of gene transfer of unconcentrated or concentrated virus. However, our results permit eliminating some possibilities: the polymers do not stimulate target cell growth (Figure 1) or eliminate the requirement for the retrovirus receptor (Figure 7).

We were surprised to find that the virus trapped in the high molecular weight complex could transduce cells and that the efficiency of gene transfer was enhanced by the complex. We expected to find that the complex might reduce gene transfer by impeding the ability of the virus to diffuse to and bind to the virus receptor on the cell surface. In addition, since both cationic and anionic polymers were used, the complex is probably neutrally charged and is therefore unlikely to enhance gene transfer simply by reducing the electrostatic repulsion between the virus and the cell, as has been previously observed when cationic polymers only were added to retrovirus stocks [9, 10, 20].

One possibility, given the selectivity with which retroviruses are incorporated into the complexes, is that complex formation enhances gene transfer by purifying or separating the viruses from an inhibitor of transduction. Another possibility is that the virus-polymer complexes are large enough to sediment onto the target cells. This would increase the rate at which viruses encounter target cells, thereby reducing the number of viruses that decay before they reach the cell by simple diffusion [21, 22]. Neither possibility is ruled out by our observation that pelleting and resuspension of the virus-polymer complex increased the titer by about three-fold over virus that had formed a complex but had not been pelleted and resuspended. For example, it is possible that the virus is tightly associated with an inhibitor that is not released by simple dilution into cell culture medium but which is released during the process of pelleting and resuspension. Alternatively, it is possible that pelleting, which substantially increases the concentration of the polymers and virus (until they are resuspended) induces a change in the size or structure of the complex that improves gene transfer.

Our results show that by adding CSC and PB, retrovirus can be rapidly concentrated and purified in a single step without destroying biological activity. The selectivity (cfu/mg protein) of the process with respect to virus activity is more than 1000-

fold. The complexes captured 80% of the virus particles in the stock, as measured by p30, but they captured 300% virus stock's gene transfer activity. Moreover, the complexes only incorporated 0.3% of the protein present in the original virus stock. Therefore, this single processing step represents a significant step in the purification of retrovirus.

Complexation of retroviruses into polymer complexes may have several important applications. It can be used to rapidly concentrate retrovirus and significantly improve the efficiency of *ex vivo* gene transfer. Given its simplicity, rapidity, and low cost, if it can be scaled-up, it may have advantages over other large-scale processing approaches such as tangential flow, hollow fiber filtration, ultrafiltration and ultracentrifugation. [23-27]. The virus-polymer complexes can be resuspended in a culture medium that is optimal for the proliferation of the intended target cell type which may help increase gene transfer. Moreover, the purification achieved by the formation of the complexes may remove inhibitors or undesirable impurities. Such purification steps are important for the eventual formulation of a therapeutic retrovirus preparation that is suitable for injection and *in vivo* gene transfer applications.

A better understanding of the mechanism by which viruses are captured into polymer complexes and the key properties of the polymers that are necessary for complex formation would permit optimizing this procedure for specific gene transfer applications. In the following chapters, we will examine in more detail the mechanism of complexation of viruses with polymers, as well as the mechanism by which virus-polymer complexes enhance gene transfer.

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CHAPTER 3

COMPLEXATION OF RETROVIRUSES WITH CHARGED POLYMERS ENHANCES GENE TRANSFER BY INCREASING THE RATE THAT VIRUSES ARE DELIVERED TO CELLS[*]

3.1 Abstract

We have previously found that retrovirus transduction is enhanced when an anionic polymer (chondroitin sulfate C) is added to virus stocks that contain an equal weight concentration of a cationic polymer (Polybrene). This observation was unexpected given that previous work has shown that cationic polymers enhance transduction while anionic polymers have the opposite effect. Using model recombinant retroviruses and lentiviruses that encode for the *Escherichia coli lacZ* gene and quantitative assays of virus adsorption and transduction, we examined the mechanism of enhancement. We found that addition of oppositely charged polymers (Polybrene and chondroitin sulfate C) to virus stocks enhanced gene transfer by increasing the flux of active viruses to the cells. Virus-polymer complexes formed that did not reduce the stability of the viruses yet were large enough to sediment, delivering the viruses to the cells more rapidly than by simple diffusion. The size of the complexes, the rate of sedimentation, and the levels of gene transfer increased with increasing concentrations of polymers. The degree to which transduction was enhanced ranged from 2 to nearly 40-fold, and varied depending on the type of cells and viruses used. Interestingly, we found that association of the viruses with the polymer complexes did not significantly hinder their ability to complete post-binding steps of transduction. We concluded that

[*] Modified from Journal of Gene Medicine, 2004, 6(12):1304-19

complexation of retroviruses with charged polymers significantly improves the efficiency of *ex vivo* gene transfer by increasing the number of active viruses that reach the cells.

3.2 Introduction

Recombinant retroviruses offer a number of advantages as gene transfer vectors, particularly when permanent modification of the genome is desired [1, 2]. Nevertheless, their success in human gene therapy clinical trials has been limited, in part because they fail to transfer enough genes to cells to achieve the desired therapeutic effect [1, 3, 4]. Retrovirus transduction appears to be limited by a number of barriers, a significant one being the slow rate that retroviruses bind to cells [5-7]. Retrovirus binding to cells is limited by biophysical constraints that control the rate that viruses are transported to the cells, and by biochemical factors that interfere with their adsorption once they have reached the surfaces of the cells.

In conventional transductions, retroviruses are transported to cells by diffusion, a slow process compared to the rate that viruses lose their bioactivity (decay). As a result, less than one-tenth of viruses used in a typical gene transfer protocol reach the surfaces of the cells before losing their bioactivity [5, 7, 8]. Viruses that successfully reach the cell surface while still active must then bind to the cell. This initial binding event appears to involve interactions between extracellular matrix proteins on the surfaces of the cells and viruses, and is strongly influenced by the presence of charged polymers in the virus stocks [9-12]. Cationic polymers enhance retrovirus binding and transduction, presumably by reducing the electrostatic repulsion between the negatively charged viruses and cells [13-17]. Anionic polymers almost always inhibit retrovirus transduction, presumably by increasing the electrostatic repulsion between the viruses and cells [12, 16, 18-20]. Not all anionic polymers inhibit transduction solely by increasing the electrostatic repulsion between the viruses and the cells. For example, we have

previously shown that chondroitin sulfate proteoglycans and glycosaminoglycans inhibit retrovirus transduction, but only if they have a high degree of sulfation and a specific carbohydrate structure [21]. Others recently observed that free heparin and heparan sulfate glycosaminoglycans inhibit retrovirus binding and transduction by competitively binding to the heparan-binding domains of fibronectin molecules bound to the surfaces of cells or tissue culture plates [22, 23] or by binding to the surface of the virus and competing with cell associated glycosaminoglycans for virus binding [19].

While studying the effects of charged polymers on transduction, we previously found that the combined addition of an anionic polymer (chondroitin sulfate C; CSC) and a cationic polymer (Polybrene; PB) led to the formation of virus-polymer complexes that contained more than 80% of the virus particles, but less than 0.3% of other proteins (refer to Chapter 2) [24]. To concentrate and purify the virus-polymer complexes, we pelleted them by low speed centrifugation, resuspended them to one-eighth their original volume in fresh medium, and then used them to transduce cells. Gene transfer was enhanced 10 to 20-fold. Interestingly, gene transfer was enhanced 3 to 4-fold even when the virus-polymer complexes were not concentrated or purified, but the mechanism of enhancement was not explored. Given these promising results, we decided to investigate the mechanism by which the polymers enhance gene transfer without the need for concentration or purification.

3.3 Materials and methods

Chemicals and antibodies. Chondroitin sulfate C (CSC) (shark cartilage), gluteraldehyde, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene, PB), o-Nitrophenyl β-D-galactopyranose (ONPG) and Igepal CA-630 were from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide 30%, and Polyoxyethylene 20-Sorbitan Monolaurate (Tween 20) were from Fisher Scientific (Fair Lawn, NJ). Non-fat

dry milk (blotting grade) was from Bio-Rad Laboratories (Hercules, CA). o-Phenylenediamine Dihydrochloride (OPD) was from Pierce (Rockford, IL). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Denville Scientific, Inc. (Metuchen, NJ). Mouse anti-p30 antibodies were purified from the supernatant of the CRL-1219 hybridoma cell line (ATCC, Rockville, MD) following standard procedures [25]. The goat polyclonal anti-p30 antibody (78S221) was from Quality Biotech (Camden, NJ). The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories (South San Francisco, CA).

Cell culture. NIH 3T3 mouse fibroblasts and the amphotropic packaging cell line Ψ-CRIP producing the α-SGC-*LacZ* virus [26], kind gifts of Jeffrey R. Morgan, and the ecotropic packaging cell line CRE BAG, obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% bovine calf serum (Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Hyclone Labs Inc.) and 110 μg/mL of sodium pyruvate (Hyclone Labs Inc.) (DMEM/BCS). TELCeB6 (TE671 cells expressing Mo-MLVgagpol and the retroviral vector MFGnlsLacZ [27] were kind gifts of F.L. Cosset. TE671 (human medulloblastoma epithelial cells), HeLa cells (human adenocarcinoma cells), and 293T/17 human embryonic kidney cells were obtained from the ATCC. These cell lines were cultured in DMEM, 10% fetal bovine serum (Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 110 μg/mL of sodium pyruvate (DMEM/FBS).

Generation of a stable amphotropic retrovirus packaging cell line. An amphotropic packaging cell line (TELCeB6-A) was generated by stable transfection of TELCeB6 cells. Five micrograms of the plasmid FB4070ASALF, an expression plasmid that encodes for the amphotropic envelope glycoprotein (a kind gift of Stephen Russell),

was dissolved in 400 μL of 0.25M $CaCl_2$, mixed with 400 μL of 2X HEPES buffered saline (274 mM NaCl, 42 mM Hepes acid, 10 mM KCl, 1.4 mM Na_2HPO_4 and 12 mM dextrose), incubated at room temperature for 20 minutes, then added to a 50% confluent T75 flask of TELCeB6 cells. Twelve hours after transfection the cells were washed with PBS, and then the medium replaced with fresh DMEM/FBS. Two days later the cells were trypsinized, pelleted, and resuspended. Two hundred microliters of the resuspended cells were diluted in 10 mL of selective medium (DMEM, 10% FBS, 50 μg/mL of phleomycin, and 7 μg/mL of blasticidin), and plated in a T75 flask. Fourteen days later pooled clones of stably transfected cells were frozen for later use.

Production of retroviruses and lentiviruses. To generate retrovirus stocks, virus-producing cells were grown to confluence in T175 tissue culture flasks, and then incubated for 24 h with 35 mL of DMEM/BCS. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use. We produced lentivirus vectors by transient transfection of 293T/17 cells that were plated the day before in 10-cm tissue culture dishes ($6x10^6$ cells/dish), and cultured in 10 mL of DMEM that contained heat inactivated FBS (10%) but no antibiotics. The next day (between 18 to 30 h later), cells were transfected with pCMVΔR8.91 (kind gift of Scott S. Case, [28]), pTY-EfnlacZ (NIH AIDS Research & Reference Reagent Program, Rockville, MD), and pMD.G (kind gift of Scott S. Case, [28]) or FB4070ASALF. To produce GFP-labeled lentivirus, cells were also transfected with GFP-Vpr (a kind gift of Thomas Hope) [29]. Eight micrograms (six micrograms for GFP-labeled lentivirus) of each plasmid were diluted into a total of 1.5 mL of DMEM, then mixed with a solution of 1.5 mL of DMEM and 90 μL of Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA), then added to the cells. Thirty-six hours later the medium was replaced with 10 mL of

DMEM/FBS. Twelve hours later the virus-laden cell culture supernatant was harvested, filter sterilized (0.45-μm) and frozen (-80°C) for later use.

Diluted titer assay. Ten-fold serial dilutions of *lacZ* virus stock were made in DMEM/BCS and Polybrene (8 μg/mL). A 1-mL amount per well was used to transduce 3T3 cells that had been seeded (7 x 10^4 per well) the previous day in a 12-well plate. Two days after the start of the transduction, the cells were fixed and stained for β-galactosidase activity with X-Gal [30]. Colonies of *lacZ*+ cells (typically in clusters of 2, 4, or 8 blue cells) were counted with the aid of a dissecting microscope. At appropriate dilutions of the virus stock, the clusters of blue cells were sufficiently spread over the dish such that each cluster arose from a single transduction event. From triplicate wells, the number of *lacZ*+ CFU per milliliter was 6.1 x $10^4 \pm$ 0.6 x 10^4 for amphotropic retrovirus produced from the Ψ-CRIP cell line, 1.1 x $10^5 \pm$ 0.2 x 10^5 for ecotropic retrovirus produced from the CRE BAG cell line, 7.2 x $10^6 \pm$ 0.6 x 10^6 for amphotropic retrovirus from the TELCeB6-A cell line, 1.0 x $10^5 \pm$ 0.06 x 10^5 for amphotropic lentivirus produced by transient transfection of 293T/17 cells, and 3.1 x $10^4 \pm$ 0.3 x 10^4 for vesicular stomatitis virus glycoprotein (VSVG) pseudotyped lentivirus produced by transient transfection of 293T/17 cells.

Beta-galactosidase (β-gal) assay. To test the effect of polymers on transduction, five thousand NIH 3T3 murine fibroblasts in 100 μL of medium were plated per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, MA). The next day (19 to 26 h later), virus stocks were brought to a range of equal weight concentrations of PB and CSC by adding, in immediate succession, appropriate volumes of aqueous stock solutions (20 mg/mL) of the polymers and vortexing the mixture. The concentration of PB was then raised an additional 8 μg/mL. The medium was removed and 100 μL of the virus-polymer mixture added to

each well. Two days after transduction, the medium was removed and the cells washed once with 100 μL of phosphate-buffered saline (PBS) containing 1mM $MgCl_2$. After removal of the wash solution, 50 μL of lysis buffer (PBS with 1mM $MgCl_2$ and 0.5% Igepal) were added to each well, and the plate incubated at 37°C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG warmed to 37°C were added to each well, and the plate incubated at 37°C for 5 to 60 min until a visible yellow color was obtained. The reactions were halted by the addition of 20 μL per well of stop buffer (1M Na_2CO_3). The optical density at 420 nm (OD_{420}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA) and the non-specific background at 650 nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.

Percent transduced assay. Cells were transduced with the *lacZ* virus as described above, grown to confluence, and trypsinized; then 20 μl of the trypsinized cells were diluted into 1 mL of medium. Ten microliters of this dilution were plated into a 10-cm dish. After 10 days, macroscopic colonies had grown and were fixed and stained for β-galactosidase activity with X-Gal as described in the diluted titer assay. Colonies were counterstained with a solution of 1% rhodamine B, rinsed with water and air-dried. Values of the percentage of lacZ+ colonies at each point are the averages of three independent experiments.

Virus incubation with polymers. Five thousand NIH 3T3 murine fibroblasts in 100 μl of DMEM/BCS were plated in each well of a 96-well flat-bottomed tissue culture dish. The next day (19 to 26 h later), the cells were incubated with virus (α-SGC-*LacZ*) and PB (8 μg/mL) for 2 h and either pretreated (Pre), cotreated (Co) or post-treated (Post) with polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC). Pretreated cells were incubated with medium that contained polymer complexes (320 μg/mL of PB

and 320 μg/mL of CSC) for 2 hours, washed three times with fresh medium (100 μL/well), then incubated for 2 hours with virus and PB (8 μg/mL). Cotreated cells were incubated with virus containing polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC) plus an additional 8 μg/mL PB, then washed three times with fresh medium. Post-treated cells were incubated for 2 hours with virus and PB (8 μg/mL), washed three times with fresh medium, incubated for 2 hours with medium containing polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC) and washed three times with fresh medium. As control, cells were incubated with virus and PB (8 μg/mL) and washed three times with fresh medium (None). All cells were then incubated with fresh medium (100 μL/well) for two days at 37°C until confluent, and then tested for β-gal activity in the β-gal assay.

Phase contrast and fluorescence microscopy. To visualize virus-polymer complexes, amphotropic *LacZ* retrovirus stocks produced from the ψ-CRIP cell line containing equal weight concentrations of PB and CSC were added to NIH 3T3 fibroblasts that had been plated the previous day at 70,000 cells per well in 12-well dishes. Phase contrast images of the complexes were taken one day later (40x). In addition, stocks of GFP-labeled amphotropic lentiviruses and fresh cell culture medium were brought to 640 μg/mL of PB and CSC, incubated overnight at 37°C, then observed under an inverted fluorescence microscope (IX-50, Olympus America, Inc., Melville, NY).

ELISA for p30. We used an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of virus capsid protein (p30) in cell lysates and virus stocks. ELISA plates (Nunc immuno Maxisorp 96-well plates, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to

block non-specific binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 was sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer, and incubated for 1 h at 37°C. The horseradish peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed for 5 min using hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Virus adsorption and degradation assay. Adsorption to cells and subsequent degradation of amphotropic retrovirus were analyzed by ELISA. NIH-3T3 cells were plated in 12-well dishes (7.0×10^4 per well) and then cultured at 37°C for 3 days until confluent. The medium was removed and replaced with 0.5 mL of virus stock brought to a range of equal weight concentrations of PB and CSC, and then the concentration of PB raised by an additional 8 μg/mL. For virus adsorption experiments, cells were incubated with virus for a range of times, then the supernatant and cell lysates analyzed for levels of p30 by ELISA. For virus degradation experiments, plates were centrifuged for 30 min at 4°C at 2500 rpm (1100 g) to establish a cohort of viruses on the surfaces of the cells (pulse), washed three times at 4°C with 1 mL of fresh culture medium (chase), incubated for a range of times, then the cell lysates analyzed for levels of p30 by ELISA.

To quantify the levels of p30 in cell lysates, cells were washed once with fresh medium, incubated with 0.2 mL lysis buffer (1% Igepal, 150mM NaCl and 50mM Tris, pH 8) containing protease inhibitor (Complete Mini, Roche Diagnostics GmbH, Germany) on ice for 30 min, then centrifuged at 14000 rpm (20000 g) for 10 min at 4°C to remove cellular debris. The resulting supernatant was stored at -80°C for later analysis by ELISA.

Measurement of retrovirus decay. Frozen *lacZ* virus in culture medium was thawed, brought to an equal weight concentration of PB and CSC (0 or 640 μg/mL), then incubated at 37°C and 10% CO_2. Samples were taken at regular intervals and frozen at -80°C. At later times, the samples were thawed, brought to 8 μg/mL of PB, and then used to transduce NIH 3T3 cells in the β-gal assay.

Estimation of the rates of virus binding and intracellular degradation. To determine rates of virus binding and intracellular degradation for a range of polymer concentrations, we assumed that the rate of change in the concentration of free virus particles (active plus heat-inactivated) (F) is described by:

$$\frac{dF}{dt} = k_{sed} F \qquad (1)$$

where k_{sed} is the virus sedimentation rate. We also assumed that once virus particles are associated with target cells, they are degraded intracellularly at a rate that is proportional to the concentration of cell-associated virus particles. The rate of change in the concentration of virus that is associated with cells (B) but not yet degraded is described by:

$$\frac{dB}{dt} = k_{sed}F - k_{\deg}B \qquad (2)$$

where $k_{\deg}$ is the intracellular degradation rate. Therefore, the concentration of undegraded virus that is associated with cells (B) as a function of time is described by the equation:

$$B = \frac{k_{sed}F_0}{k_{\deg} - k_{sed}}(e^{-k_{sed}t} - e^{-k_{\deg}t}) \qquad (3)$$

where F_0 is the concentration of free virus at the start of transduction. As one of two independent means to estimate the values of the degradation and sedimentation rate constants, we used nonlinear regression of data from Figure 3.7 to equation (3) to find their best fit-values.

The rate of change in the concentration of virus that is associated with cells (B) but has not yet degraded during a pulse-chase experiment is described by the equation:

$$\frac{dB}{dt} = -k_{\deg}B \qquad (4)$$

This equation is similar to equation (2), except that for a pulse-chase experiment, the rate of virus binding is zero because the cell culture medium (chase) does not contain any virus (i.e, F = 0). As a second independent means to estimate the value of the degradation rate constant, we found its best-fit value using equation (4) and data from Figure 3.10.

Estimation of the efficiency of post-binding steps of transduction. To determine the efficiency of post-binding steps of transduction, the efficiency of gene transfer (i.e., β-galactosidase activity) for a range of polymer concentrations was plotted versus the number of active viruses to reach the cells, which we reported as a fraction of the total number of active viruses that were present at the start of the transduction. The efficiency of post-binding steps of transduction was assumed to be proportional to the best-fit slopes of these curves, which were determined using the method of least squares. The efficiency of gene transfer was measured using the β-gal assay and the number of active viruses to reach the cells was determined using a simple mathematical model of virus sedimentation and decay (described below).

We assumed that active viruses bind to cells at a rate that is proportional to the concentration of active particles in solution (V). Therefore, the rate that active viruses reach the cells is described by:

$$\frac{dC}{dt} = k_{sed} V \tag{5}$$

where k_{sed} is the virus sedimentation rate and C is the cumulative concentration of viruses to have reached the cells in an active state. The fraction (A) of the total number of active viruses present at the start of transduction (V(t=0)) to reach the cells is described by:

$$A(t) = \frac{C(t)}{V(t=0)} \tag{6}$$

Equation (6) was evaluated for t = 48 h, the length of a typical transduction. To solve equations (5) and (6), values for k_{sed} and V were needed. As a second independent means to estimate the value of the sedimentation rate constant for each polymer concentration, we used the method of least squares to fit experimental data that measured the disappearance of p30 from solution (see Figure 3.8) to equation (1) where F is the concentration of p30 in solution as measured by ELISA. The concentration of active virus in solution (V) was assumed to vary with time due to decay and sedimentation as follows:

$$\frac{dV}{dt} = -(k_d + k_{sed})V \qquad (7)$$

where k_d is the virus decay rate. The best-fit value for the virus decay rate (k_d), 8 hours, was determined by using the method of least squares to fit experimental data for decay (Figure 3.13) to the following equation:

$$\frac{dV}{dt} = -k_d V \qquad (8)$$

as previously described [5].

Data analysis. Data are summarized as the mean +/- the standard deviation for at least triplicate samples. Statistical analysis was performed using one-way analysis of variance for repeated measurements of the same variable. The Tukey multiple comparison test was used to conduct pairwise comparisons between means. Differences at $P < 0.05$ were considered statistically significant. Data were fit to equations by the method of least squares or nonlinear regression.

3.4 Results

In a previous study, we found that retrovirus transduction is enhanced by the combined addition of equal weight concentrations (80 μg/mL) of a cationic polymer (Polybrene) and an anionic polymer (chondroitin sulfate C), and that cell proliferation is not affected by the polymers, even when they are used at high doses (640 μg/mL) (refer to Chapter 2) [24]. As a first step towards understanding the mechanism by which Polybrene (PB) and chondroitin sulfate C (CSC) enhance transduction, we measured the effects on transduction of changes in the concentration of the two polymers. We brought stocks of a replication-defective amphotropic retrovirus that encoded for β-galactosidase and which was harvested from the ψ-CRIP packaging cell line (α-SGC-*lacZ* virus) to a range of concentrations of CSC (0 to 1000 μg/mL) and PB (8 to 1008 μg/mL), then used them to transduce NIH 3T3 murine fibroblasts. In each experiment we used concentrations of PB that were 8 μg/mL higher than CSC in order to simplify comparisons with control experiments that used virus stocks supplemented with PB (8 μg/mL) only. As additional controls, we brought virus stocks to a range of concentrations of PB only (8 to 1008 μg/mL) or to 8 μg/mL of PB and a range of concentrations of CSC (0 to 1000 μg/mL), and then used them to transduce NIH 3T3 cells. The efficiency of gene transfer decreased substantially when high concentrations of only one polymer were used (Figure 3.1). In contrast, gene transfer increased with increasing concentrations of polymers when high concentrations of both polymers were added to the virus stock, reached a maximum enhancement when 320-640 μg/mL of CSC and 328-648 μg/mL of PB were used, then decreased slightly at the highest doses of polymers tested (Figure 3.1, Table 3.1). We confirmed these results using an assay in which the percentage of *lacZ*+ cells after transduction was determined (Figure 3.2).

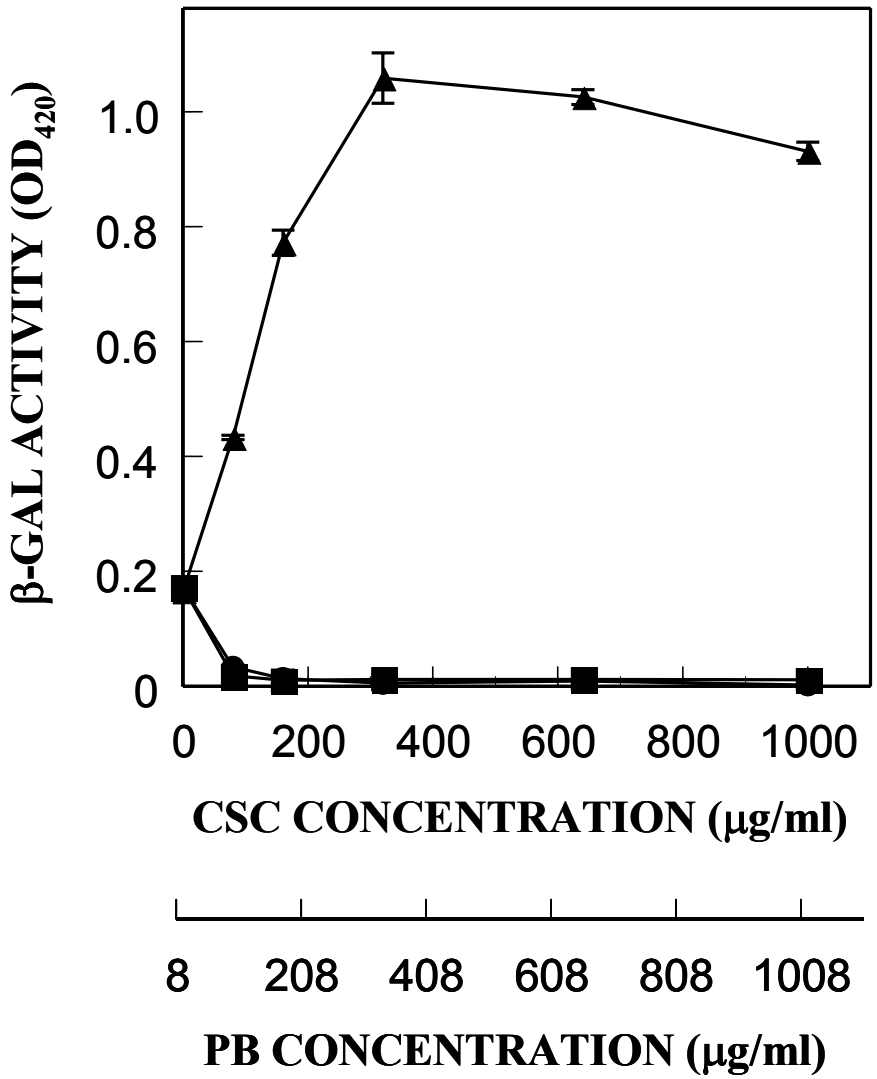

Figure 3.1 Mixtures of PB and CSC enhance gene transfer. NIH 3T3 cells were plated in a 96-well dish (5,000 per well) and, the next day, transduced with 100 μl of amphotropic *lacZ* retrovirus (α-SGC-*LacZ*) produced from the ψ-CRIP cell line that contained 0 to 1000 μg/mL CSC and a weight concentration of PB that was 8 μg/mL more than that of CSC (▲), 0 to 1000 μg/mL CSC and 8 μg/mL PB (●), or 8 to 1008 μg/mL of PB only (■). Two days later, the level of gene transfer was quantified using the β-gal assay. Each point shows the mean ± standard deviation of three replicates.

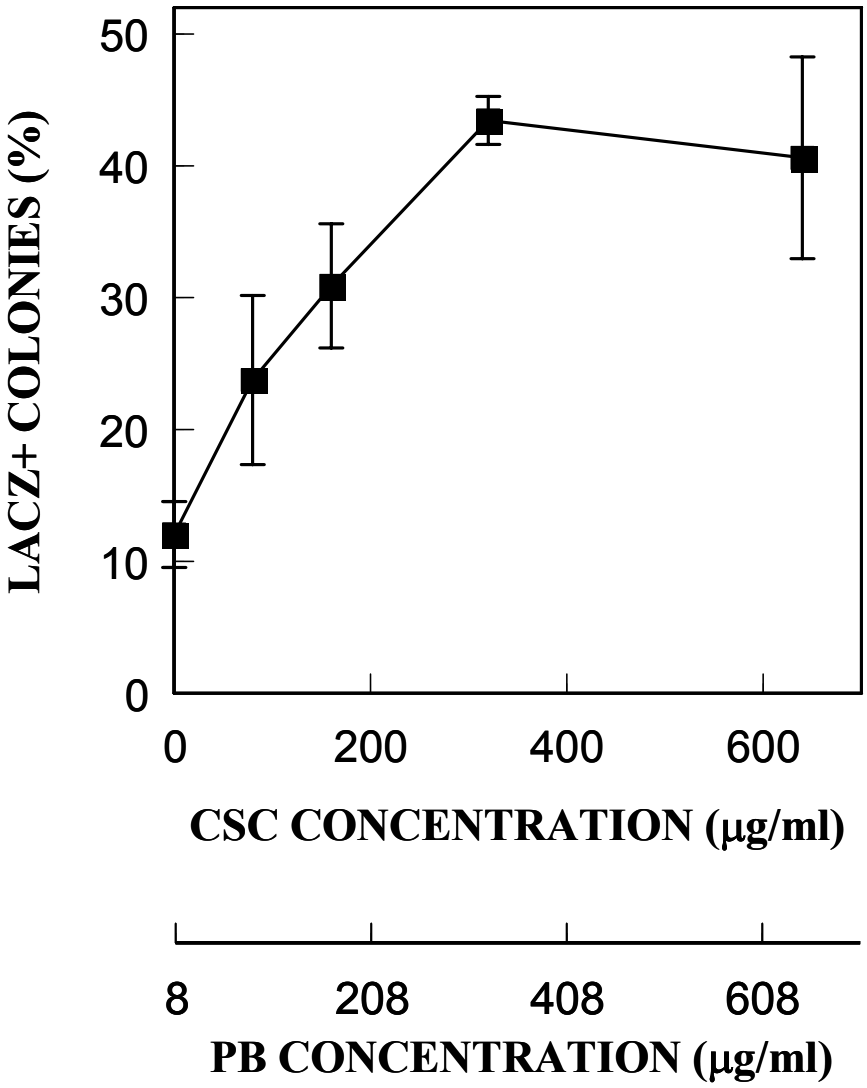

Figure 3.2 Mixtures of PB and CSC increase the percentage of cells transduced. NIH 3T3 cells were transduced with α-SGC-*LacZ* retrovirus that contained 0 to 1000 μg/mL CSC and a weight concentration of PB that was 8 μg/mL more than that of CSC. Cells were trypsinized, diluted, and plated onto a 10-cm dish. After 10 days of growth, macroscopic colonies were fixed, stained for *lacZ* activity with the X-Gal substrate, and counterstained with rhodamine B. Values for the percentage of *lacZ*+ colonies at each point (blue colonies/total colonies x 100) are the mean ± standard deviation of triplicate wells of the 96-well dish.

Table 3.1 Mixtures of PB and CSC increase the efficiency of gene transfer in a number of systems[a]

Virus Producer Cells	Virus Produced[b]	Envelope Protein	Cell Line Transduced	Polymer Dose at Max TE (μg/ml)	Fold Enhancement[c]
ψ-CRIP	MLV	amphotropic	NIH 3T3	320-640	5.5 ± 2.2
ψ-CRIP	MLV	amphotropic	Hela	320-640	37 ± 27
ψ-CRIP	MLV	amphotropic	TE671	320-640	17 ± 5.0
CRE BAG	MLV	ecotropic	NIH 3T3	320-640	13 ± 4.7
TELCeB6-A	MLV	amphotropic	HeLa	320-640	1.9 ± 0.33
293T/17	Lentivirus	amphotropic	NIH 3T3	160-1000	3.0 ± 0.80
293T/17	Lentivirus	VSVG	NIH 3T3	320-1000	3.2 ± 1.1

[a] MLV: Moloney murine leukemia virus; VSVG: vesicular stomatitis virus; TE: transduction efficiency.
[b] MLV was produced from stable virus producer cell lines; Lentivirus was produced by transient transfection of 293T/17 cells with pCMVΔR8.91, pTY-EfnlacZ, and FB4070ASALF or pMD.G.
[c] Fold Enhancement of transduction was defined as the ratio of the maximum TE achieved when mixtures of PB and CSC were used to the TE achieved when only PB (8 μg/ml) was used.

To determine if the combined addition of PB and CSC enhances the level of gene transfer to other cell lines or with different retroviruses, we brought stocks of α-SGC-*lacZ* virus to a range of CSC and PB concentrations and used them to transduce HeLa and TE671 cells (Table 3.1). We also examined the ability of the polymers to enhance the efficiency of transduction of four additional types of recombinant retroviruses: an amphotropic retrovirus produced from TELCeB6 cells, an ecotropic retrovirus harvested from the CRE BAG cell line, and lentiviruses produced by transient transfection of 293T/17 cells and pseudotyped with either the amphotropic or the VSVG envelope protein. In each case we found that the combined addition of polymers to virus stocks enhanced the efficiency of gene transfer, although the level of enhancement was somewhat variable (Table 3.1).

As a first step towards understanding the mechanism of enhancement, we tested if the polymers enhanced transduction by binding to the target cells prior to virus adsorption. NIH 3T3 cells were incubated for two hours with CSC (320 μg/mL) and PB (320 μg/mL), washed to remove unbound polymers, then exposed for two hours to α-SGC-*lacZ* virus supplemented with PB (8 μg/mL). As a control, cells that had not been pretreated with PB and CSC were transduced with virus that contained only PB (8 μg/mL). Pretreatment of cells with PB and CSC had no effect ($p < 0.05$) on transduction (Figure 3.3). To determine if polymers enhance transduction by interacting with the viruses, or by interacting with the cells and enhancing transduction after it begins, we compared the efficiency of transduction of NIH 3T3 cells incubated with α-SGC-*lacZ* virus in the presence of CSC (320 μg/mL) and PB (328 μg/mL) to the efficiency of transduction of cells that were incubated with virus supplemented with PB (8 μg/mL) only, but then incubated with both CSC (320 μg/mL) and PB (320 μg/mL) immediately following their exposure to virus. As shown in Figure 3.3, transduction was substantially

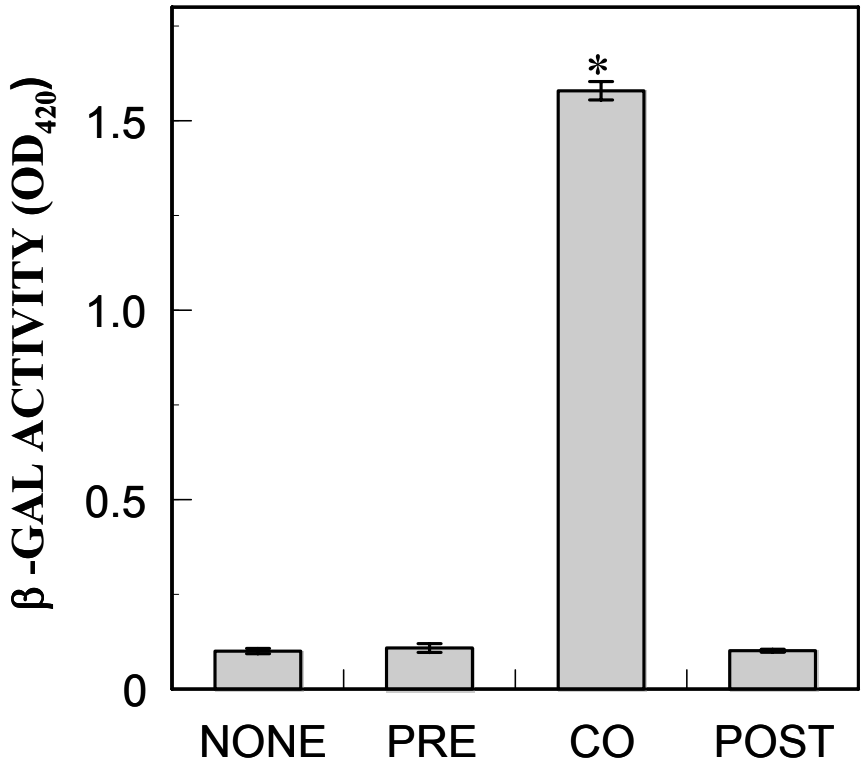

Figure 3.3 CSC and PB enhance transduction only when they are present at the same time the cells are exposed to virus. Cells, plated the previous day at 5000 cells per well in a 96 well plate, were incubated with α-SGC-*LacZ* virus and PB (8 μg/mL) for 2 h and either pretreated (Pre), cotreated (Co) or post-treated (Post) with polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC). Pretreated cells were incubated with medium that contained polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC) for 2 hours, washed three times with fresh medium, then incubated for 2 hours with virus and PB (8 μg/mL). Cotreated cells were incubated with virus containing polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC) plus an additional 8 μg/mL PB, then washed three times with fresh medium. Post-treated cells were incubated for 2 hours with virus and PB (8 μg/mL), washed three times with fresh medium, incubated for 2 hours with medium containing polymer complexes (320 μg/mL of PB and 320 μg/mL of CSC) and washed three times with fresh medium. As a control, cells were incubated with virus and PB (8 μg/mL) and washed three times with fresh medium (None). All cells were then incubated with fresh medium, grown to confluence, and tested for β-gal activity. Fold increases in β-gal activity with respect to None are plotted as the mean ± SD of three replicates. Statistically significant differences ($p < 0.05$) from None are denoted with an asterisk.

enhanced only when CSC and PB were present at the same time the cells were exposed to the virus particles ($p < 0.05$).

Shortly after transduction was initiated we observed the rapid accumulation of granular precipitates on the cells, the median size of which became larger as the concentration of polymers used increased (Figures 3.4 and 3.5). Taken together, these observations prompted us to hypothesize that the addition of CSC and PB to virus stocks leads to the formation of virus-polymer complexes that, due to their large size, rapidly sediment to the bottom of the tissue culture plates, increasing the rate of virus adsorption to the cells. To assess the possibility that virus particles were incorporated into these visible precipitates, we brought stocks of GFP-labeled lentiviruses, or fresh medium as a control, to 640 μg/mL of PB and CSC, incubated these solutions overnightat 37°C, and then visualized them by fluorescence microscopy. Viruses were associated with the complexes, and, interestingly, located primarily on their outer surfaces (Figure 3.6).

To examine if CSC and PB increase the rate of virus adsorption, we incubated confluent monolayers of NIH 3T3 cells with α-SGC-*lacZ* virus stocks that contained a range of concentrations of PB and CSC. At various times we washed and lysed the cells, then quantified the levels of cell-associated virus using an ELISA for p30, the virus capsid protein. Levels of cell-associated p30 increased rapidly within the first 4 hours of transduction, reached a plateau during the next 6 to 10 hours, then declined slightly. For each time point, the levels of cell-associated p30 increased with increasing concentrations of polymers used (Figure 3.7). These findings indicate that the combined addition of CSC and PB to virus stocks increases the rate of virus adsorption to cells, and to an extent that correlates with the concentration of polymers used.

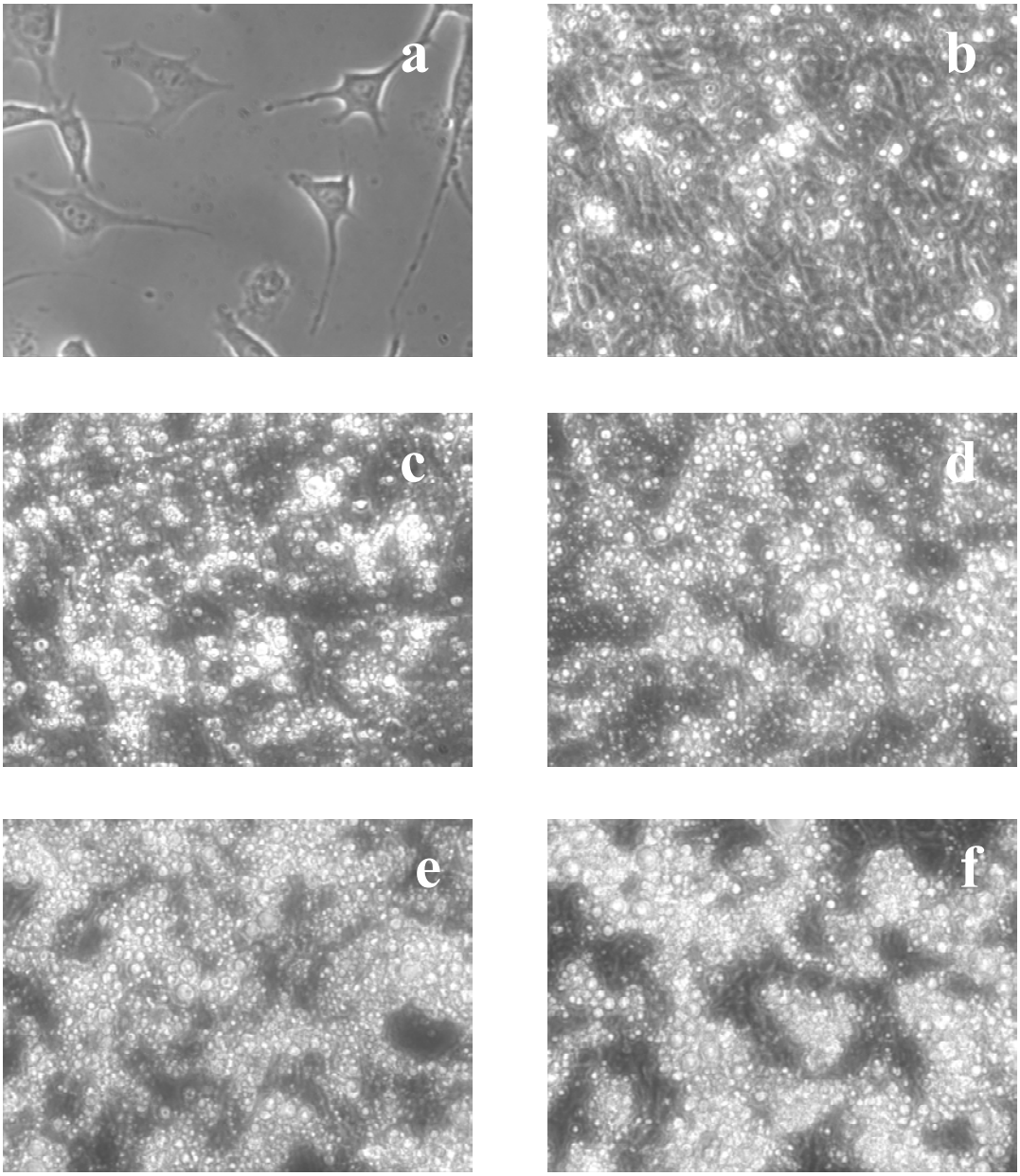

Figure 3.4 Retrovirus-polymer complexes are visible on the surfaces of cells shortly after the start of transduction. Retrovirus stocks (α-SGC-*LacZ*) were brought to 320 μg/mL of PB and CSC, and then incubated with NIH 3T3 cells for (a) 0, (b) 0.5, (c) 2, (d) 4, (e) 8, or (f) 24 h, after which the medium was removed and the cells visualized by phase contrast microscopy (40x).

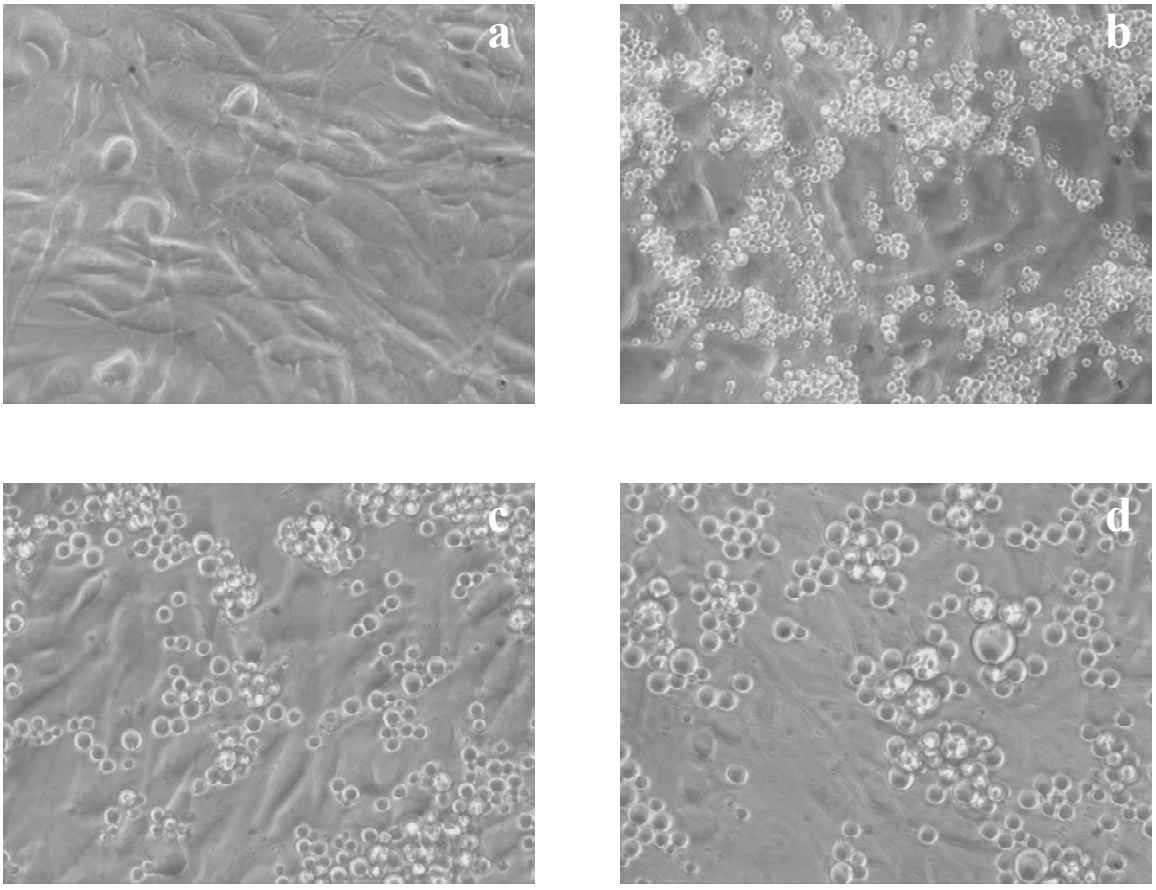

Figure 3.5 The size of the complexes increases with increasing concentrations of the polymers. Retrovirus stocks (α-SGC-*LacZ*) were brought to (a) 0 (b) 320 (c) 640 or (d) 1000 μg/mL each of PB and CSC, then incubated with NIH 3T3 cells. Phase contrast images of the complexes and cells were taken one day later (40x).

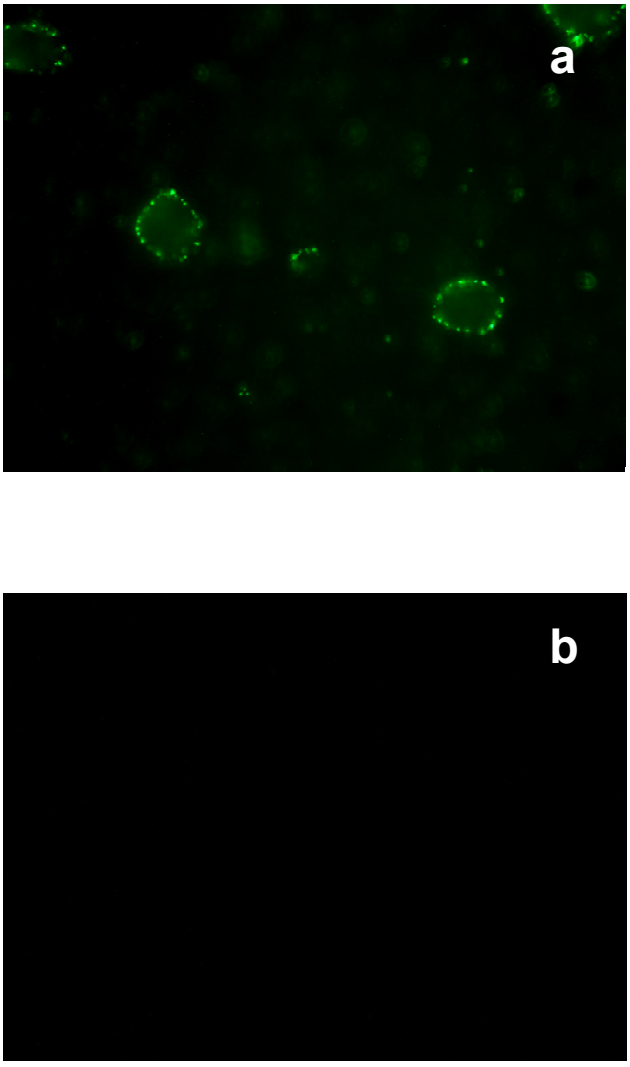

Figure 3.6 Viruses are mostly at the surface of the complexes. (a) Stocks of GFP-labeled lentiviruses were brought to 640 μg/mL of PB and CSC, incubated overnight at 37°C, and then visualized by fluorescence microscopy (magnification 60x). (b) Polymer complexes formed in fresh medium only, in the absence of viruses, did not exhibit any detectable fluorescence.

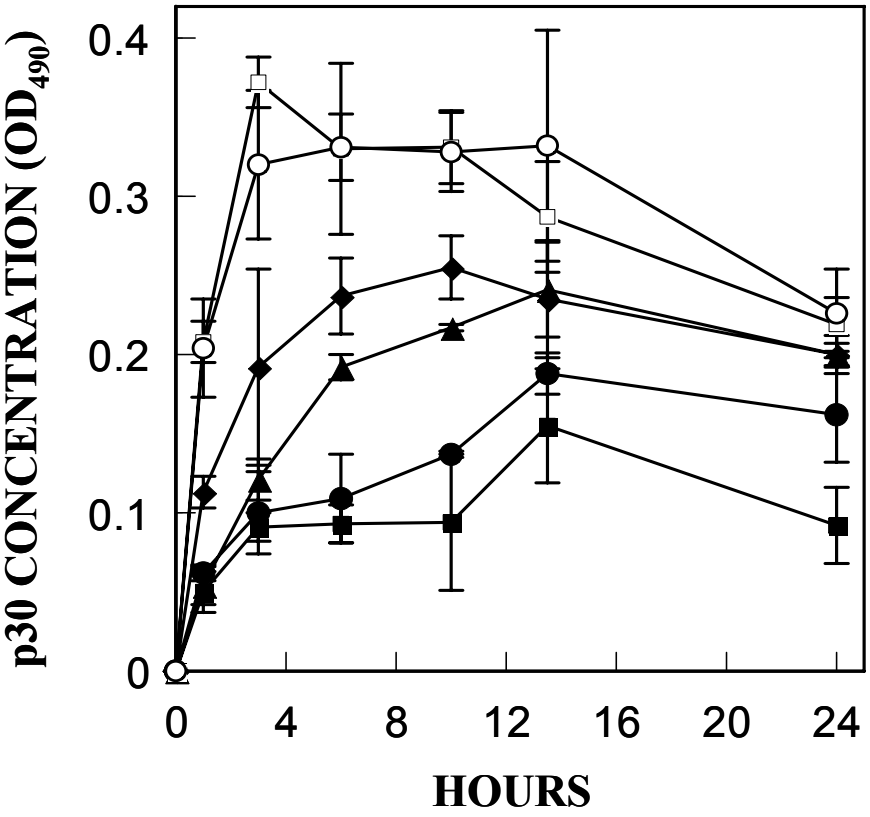

Figure 3.7 CSC and PB increase the rate of virus binding. Retrovirus stocks (α-SGC-*LacZ*) were brought to 8 μg/ml PB (■), 80 μg/ml CSC and 88 μg/ml PB (●), 160 μg/ml CSC and 168 μg/ml PB (▲), 320 μg/ml CSC and 328 μg/ml PB (◆), 640 μg/ml CSC and 648 μg/ml PB (□), and 1000 μg/ml CSC and 1008 μg/ml PB (○), then added to confluent cultures of NIH-3T3 cells. At various times the cells were lysed, and the lysates analyzed using the p30 ELISA. Each point shows the mean ± standard deviation of three replicates.

The development of a plateau in the level of cell-associated p30 at later times suggests that the rate of virus association has declined or the rate of virus removal from the cells has increased, either by dissociation or by intracellular degradation. To determine what factors contributed most significantly to the development of the plateau in cell-associated virus, we first sought to determine whether or not the rate of virus adsorption declined with time during transduction. To examine if the cell culture medium is depleted of virus particles within the first few hours of transduction, we incubated α-SGC-*lacZ* virus stocks containing PB and CSC with confluent cultures of NIH 3T3 cells, then quantified the levels of p30 in the medium at various times by ELISA. We found that when both CSC and PB were added to virus stocks, the concentration of p30 in the supernatant declined rapidly during the first 12 to 15 hours, then more slowly at later times (Figure 3.8). In addition, we found that the rate of decline increased with increasing concentrations of polymers. When only PB (8 μg/mL) was added to virus stocks, the levels of p30 declined about 15% within the first 4 hours, whereas when high doses of CSC (1000 μg/mL) and PB (1008 μg/mL) were used, the levels of p30 declined more than 70%. These findings show that, upon addition of CSC and PB, virus particles are rapidly depleted from the cell culture medium.

To examine if there were any differences between the rate at which inactive viruses are depleted from the medium versus the rate at which active viruses are depleted, we heat-inactivated stocks of α-SGC-*lacZ* virus by incubating them at 37°C for 24 hours, brought them to equal weight concentrations of CSC and PB (80, 160, or 640 μg/mL of each polymer), then incubated them with confluent cultures of NIH 3T3 cells. In parallel, we conducted the same experiment with stocks of viruses that had not been heat-inactivated. At various times we collected samples of the supernatant and measured the concentration of p30 that remained in solution. We found that the

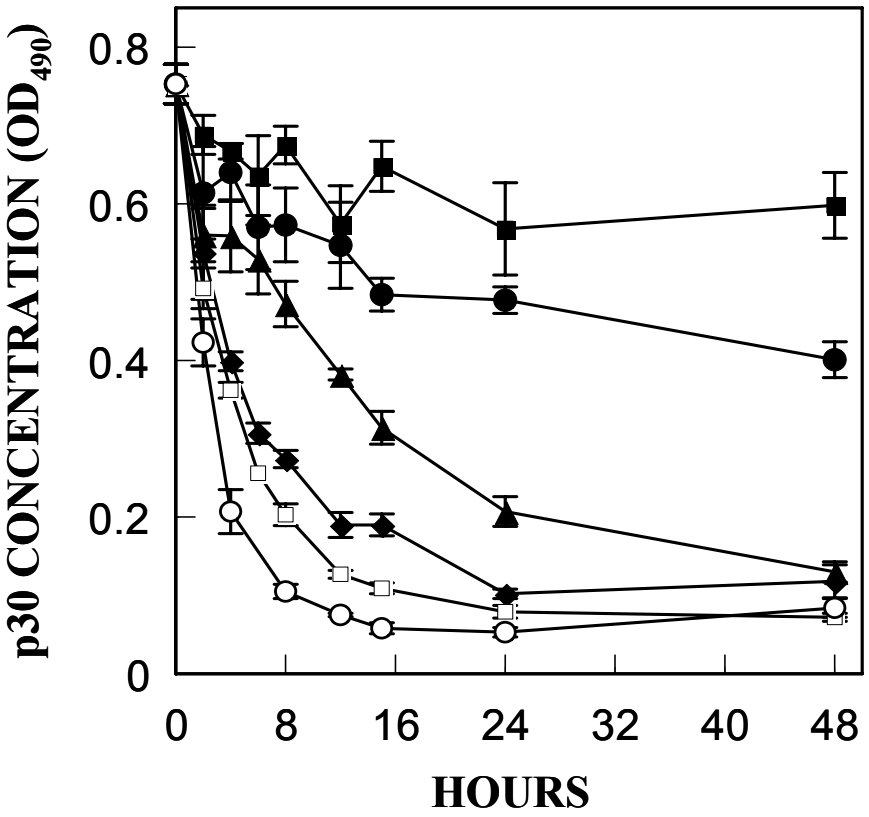

Figure 3.8 CSC and PB increase the rate of disappearance of p30 from solution. Retrovirus stocks (α-SGC-*LacZ*) were brought to 8 μg/ml PB (■), 80 μg/ml CSC and 88 μg/ml PB (●), 160 μg/ml CSC and 168 μg/ml PB (▲), 320 μg/ml CSC and 328 μg/ml PB (◆), 640 μg/ml CSC and 648 μg/ml PB (□), and 1000 μg/ml CSC and 1008 μg/ml PB (○), then added to confluent cultures of NIH-3T3 cells. At various times, the virus supernatant was collected and analyzed using the p30 ELISA. Each point shows the mean ± standard deviation of three replicates.

concentration of p30 in the supernatant declined with time and at the same rate, whether or not the particles were active or inactive (Figure 3.9). Taken together, these results suggest that it is the rapid depletion of virus particles from the cell culture medium, and not the inability of inactive viruses to sediment onto the cells, that contributes to the development of a plateau in cell-associated levels of p30 during transduction.

An increase in the rate of removal of cell-associated p30 from cells, either by degradation of internalized viruses or by the release of bound viruses from the cell surface, could also contribute to the development of a plateau in the levels of cell-associated p30. To examine these two possibilities, we performed a pulse-chase experiment in which α-SGC-*lacZ* virus stocks were brought to a range of concentrations of CSC and PB, centrifuged onto NIH 3T3 cells at 4°C for 30 minutes at 1100 g (pulse), then removed and replaced with fresh medium (chase) that had been pre-warmed to 37°C. During the chase period (12 h), we monitored the cell culture medium and cell lysates for levels of p30. We did not detect any p30 in the medium during the entire chase period (data not shown). Levels of cell-associated p30 dropped significantly during the chase period at a rate that correlated with the levels of cell-associated p30. Levels of p30 declined most rapidly at early times when the levels of p30 were high and least rapidly at later times when the levels of p30 were low (Figure 3.10). The development of a plateau in the levels of cell-associated p30 during transduction, following an initial rapid rise, can therefore be explained as the result of a decrease in the rate at which new p30-containing virus particles reach the cell surface due to depletion of viruses from the culture medium, coupled with an increase in the rate at which p30 is degraded due to its accumulation to higher levels within the cells over time. We utilized the measurements of association of p30 with target cells (Figure 3.7) and a simple mathematical model (see Materials and Methods) to estimate the rates of

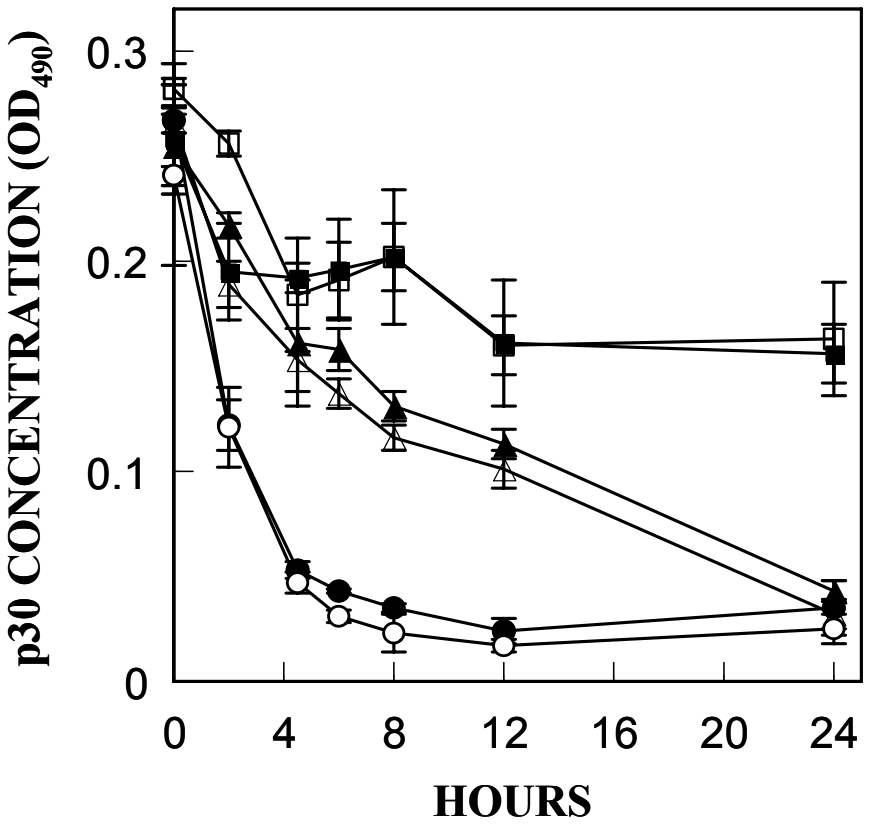

Figure 3.9 Bioactive and heat-inactivated viruses bind to the cells at the same rate. Retrovirus stocks were divided into two aliquots; one was heat-inactivated by incubation at 37°C for 24 h, then frozen, whereas the control aliquot was frozen immediately. Active (closed symbols) and heat-inactivated (open symbols) virus stocks were thawed, brought to 80 μg/ml CSC and 88 μg/ml PB (active (■), inactive (□)), 160 μg/ml CSC and 168 μg/ml PB (active (▲), inactive (Δ)), and 640 μg/ml CSC and 648 μg/ml PB (active (●), inactive (○)), then added to confluent cultures of NIH 3T3 cells. At various times, samples of the supernatant were taken and analyzed for virus concentration with the p30 ELISA. Each point shows the mean ± standard deviation of three replicates.

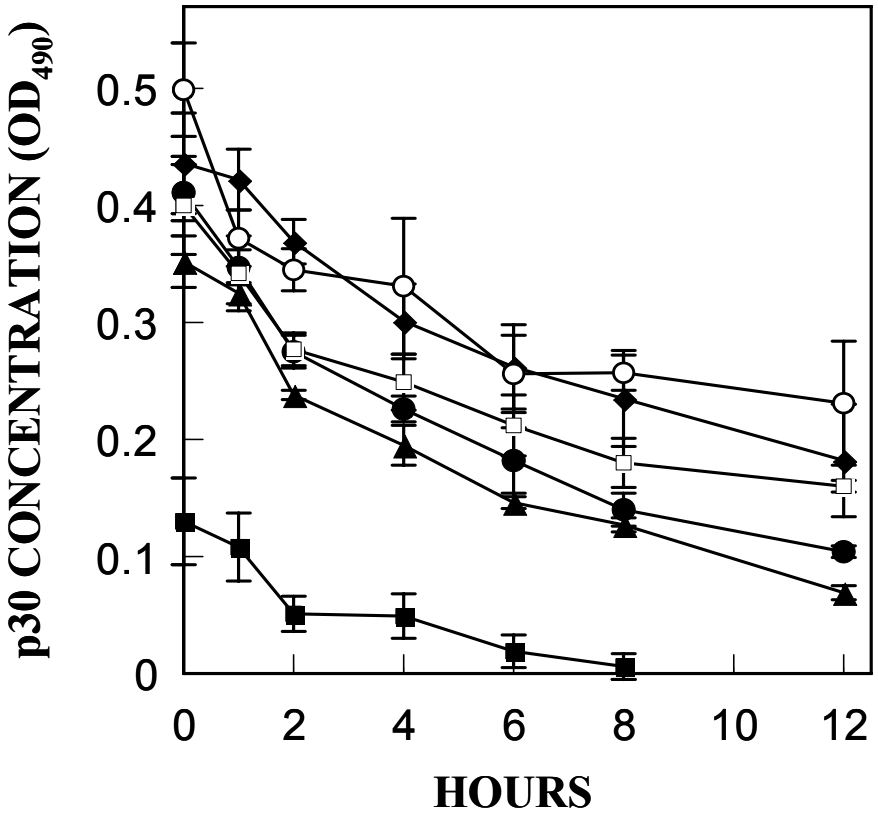

Figure 3.10 Intracellular degradation of viral proteins. Retrovirus stocks (α-SGC-*LacZ*) were brought to 8 μg/ml PB (■), 80 μg/ml CSC and 88 μg/ml PB (●), 160 μg/ml CSC and 168 μg/ml PB (▲), 320 μg/ml CSC and 328 μg/ml PB (◆), 640 μg/ml CSC and 648 μg/ml PB (□), and 1000 μg/ml CSC and 1008 μg/ml PB (○), then added to confluent cultures of NIH-3T3 cells. After addition of the virus stocks to the cells, the tissue culture dishes were centrifuged (30 min, 1100g, 4°C) to establish a cohort of viruses on the surfaces of the cells (pulse), washed three times at 4°C with fresh medium (chase), incubated for a range of times, then the cells lysed and the concentration of p30 in the lysates quantified by ELISA. Each point shows the mean ± standard deviation of three replicates.

binding and intracellular degradation of viral proteins (Figures 3.11-3.12). We found that the rates of binding increased with increasing concentrations of polymers while the rates of degradation did not correlate with the concentration of polymers. Degradation occurred more rapidly when only 8 μg/mL of PB were used, but remained unchanged when concentrations of PB and CSC equal to, or higher than, 80 μg/mL were used. In order to assess the validity of relying on disappearance of p30 from the supernatant to measure binding, and of performing a pulse-chase experiment to measure intracellular degradation, we also measured these rates using separate sets of data (see the Materials and Methods and Figures 3.8 and 3.10). The values for the degradation and sedimentation rate constants were similar, regardless of which method we used to estimate them (Figures 3.11-3.12).

Our results suggest that the combined addition of CSC and PB to virus stocks results in the formation of virus-polymer complexes that sediment, rather than passively diffuse, towards the surfaces of the cells, increasing their rate of delivery. These increases in the rates of virus delivery to cells would be expected to increase the efficiency of transduction, but only if the polymers did not adversely affect virus function or the successful completion of post-binding steps of transduction.

To determine if the polymers affect the rate of virus decay, stocks of α-SGC-*lacZ* virus were brought to equal weight concentrations of PB and CSC (640 μg/mL), then incubated for several hours in a tissue culture dish at 37°C. As a control, virus stocks were incubated at 37°C in the absence of polymers. At various times, samples of the virus solutions were collected, used to transduce NIH 3T3 cells, and the level of gene transfer quantified using the β-gal assay. The transduction efficiency of the virus stocks decreased with a half-life of 7 to 8 hours in the presence or absence of polymers, suggesting that the polymers do not affect the stability of the viruses (Figure 3.13).

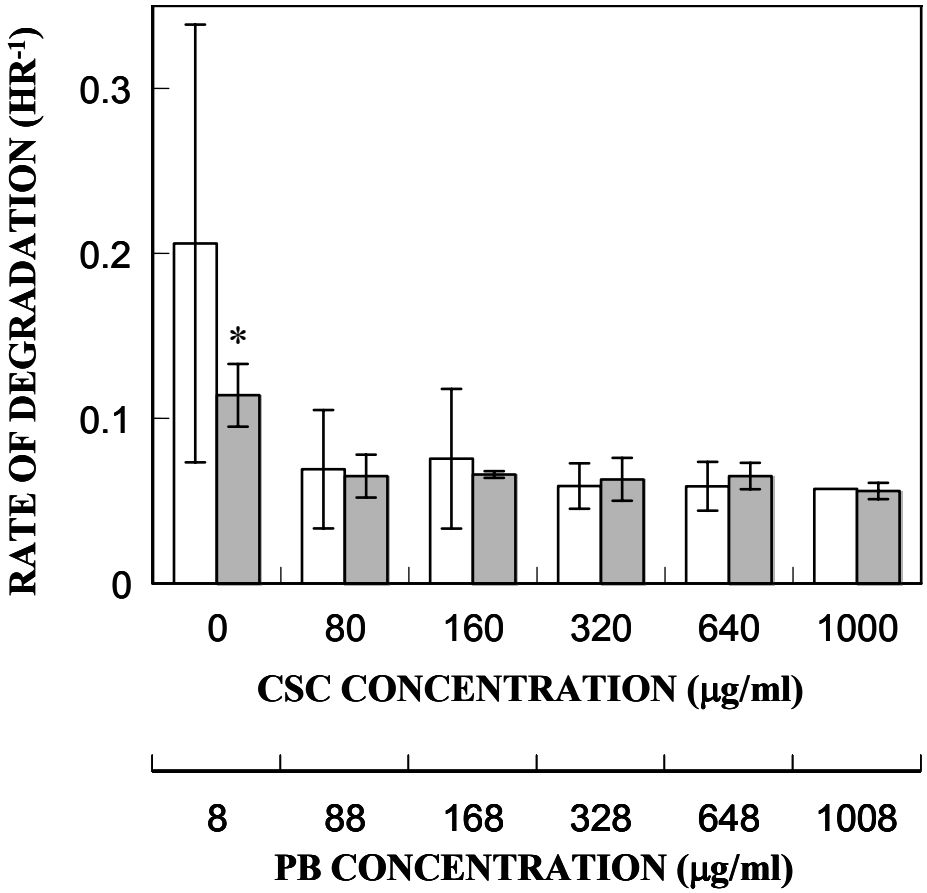

Figure 3.11 Retrovirus binding increases with increasing polymer concentration. Virus degradation rates were estimated using a mathematical model and measurements of association of p30 with target cells over time (white bars) or measurements of degradation obtained from pulse-chase experiments (gray bars). Statistically significant differences ($p < 0.05$) from the degradation rate at 80 μg/ml are denoted with an asterisk.

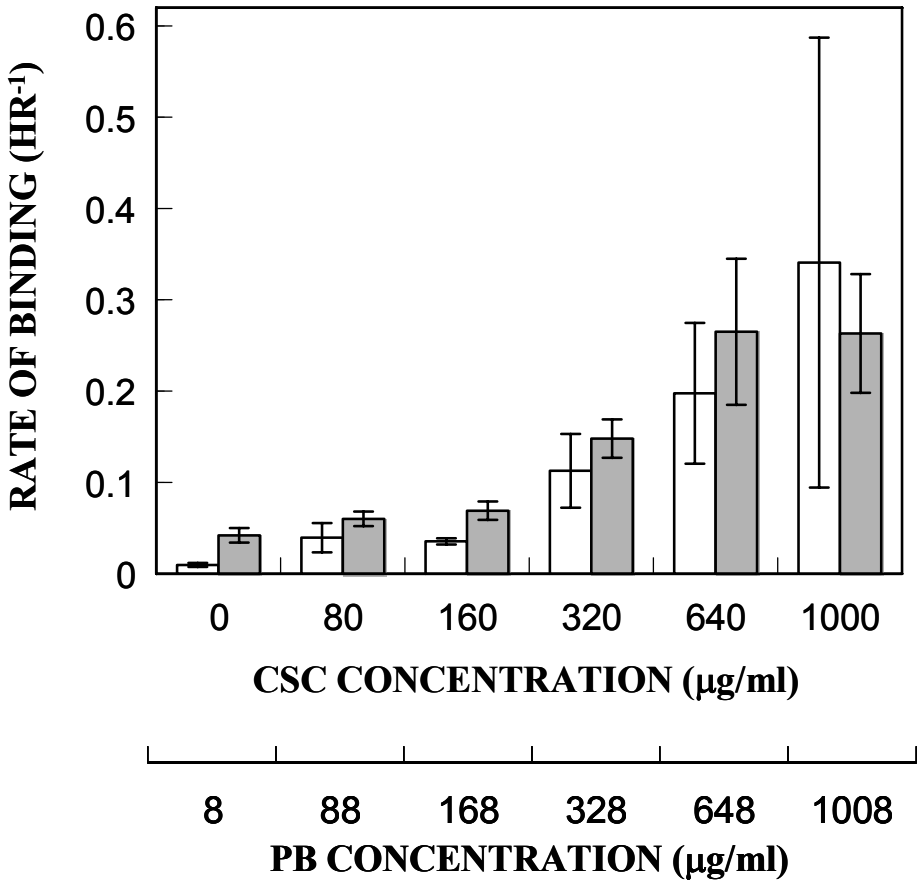

Figure 3.12 Retrovirus degradation does not correlate with the concentrations of polymers. Binding rates were estimated using a mathematical model and measurements of the concentration of p30 associated with the target cells (white bars) or the concentration of p30 remaining in the supernatant (gray bars) as a function of time.

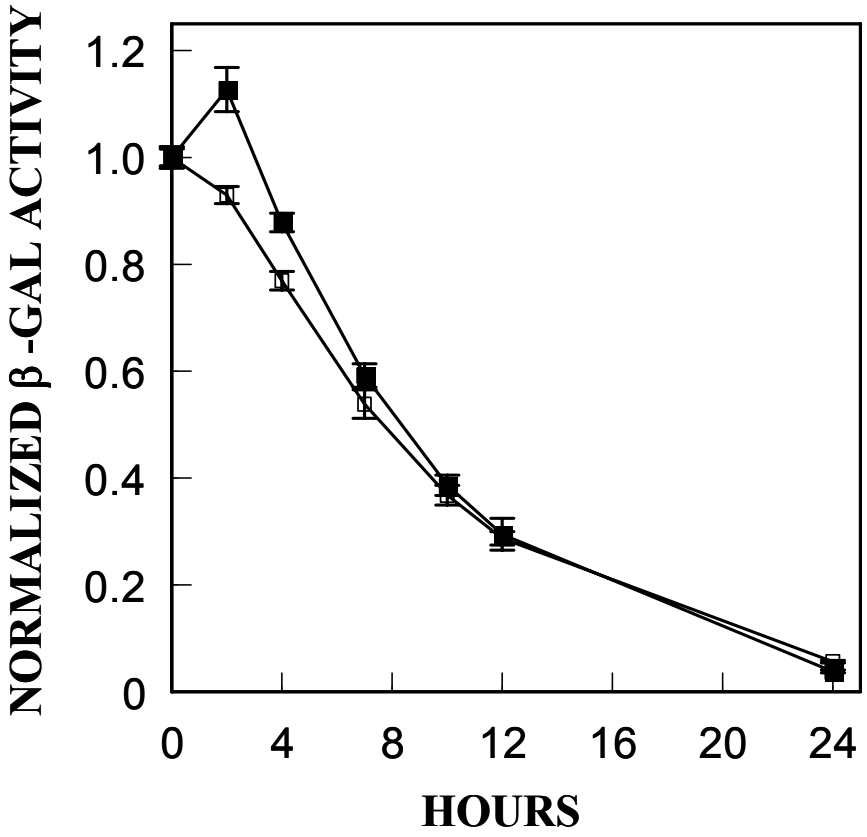

Figure 3.13 Retrovirus decay rate is not affected by the presence of CSC and PB. Retrovirus stocks (α-SGC-*LacZ*) that contained 640 μg/mL PB and 640 μg/mL CSC (□), or no polymers (■), were incubated at 37°C for 0 to 24 h. Samples were taken at various times, frozen, later thawed and their concentration of PB raised by 8 μg/mL, and then used to transduce NIH 3T3 cells in the β-gal assay. Each point shows the mean ± standard deviation of three replicates.

To determine if association of viruses with polymer complexes impedes their ability to complete post-binding steps of transduction, we compared the rate that active viruses adsorb to cells versus the rate they transduce them. To measure the transduction rate, NIH 3T3 cells were exposed to α-SGC-*lacZ* virus stocks that contained a range of concentrations of CSC (0 to 1000 μg/mL) and PB (8 to 1008 μg/mL). At intervals after the start of transduction, the virus was removed and replaced with fresh medium. Incubation continued at 37°C for two days, then the efficiency of gene transfer was quantified with the β-gal assay. The level of gene transfer increased with exposure time to virus for all solutions tested and approached a maximum after 24 h (Figure 3.14). Consistent with our previous results, the level of gene transfer increased with increasing concentrations of polymers, reached a maximum when 640 μg/mL of CSC and 648 μg/mL of PB were used, then decreased slightly when higher doses of polymers were used. Interestingly, the use of high doses of polymers seemed to accelerate the rate of transduction at early times (Figure 3.15). Half-maximal levels of transduction were reached within 2 to 3 hours when high doses (≥ 320 μg/mL) of polymers were used but only after about 6 to 7 hours when low doses (0 to 160 μg/mL) of polymers were used.

To estimate the rate that active viruses adsorb to cells in the presence of polymers, we used a simple mathematical model (see Materials and Methods). We assumed that the rate that active virus particles adsorbed to cells was proportional to the rate that they were depleted by sedimentation from the cell culture medium. We modeled virus decay using first order reaction kinetics with a half-life of 8 hours. We then plotted the level of gene transfer (i.e., β-galactosidase activity) for a range of polymer concentrations versus the number of active particles that adsorbed to the cells, which we reported as a fraction of the total number of active viruses that were present at

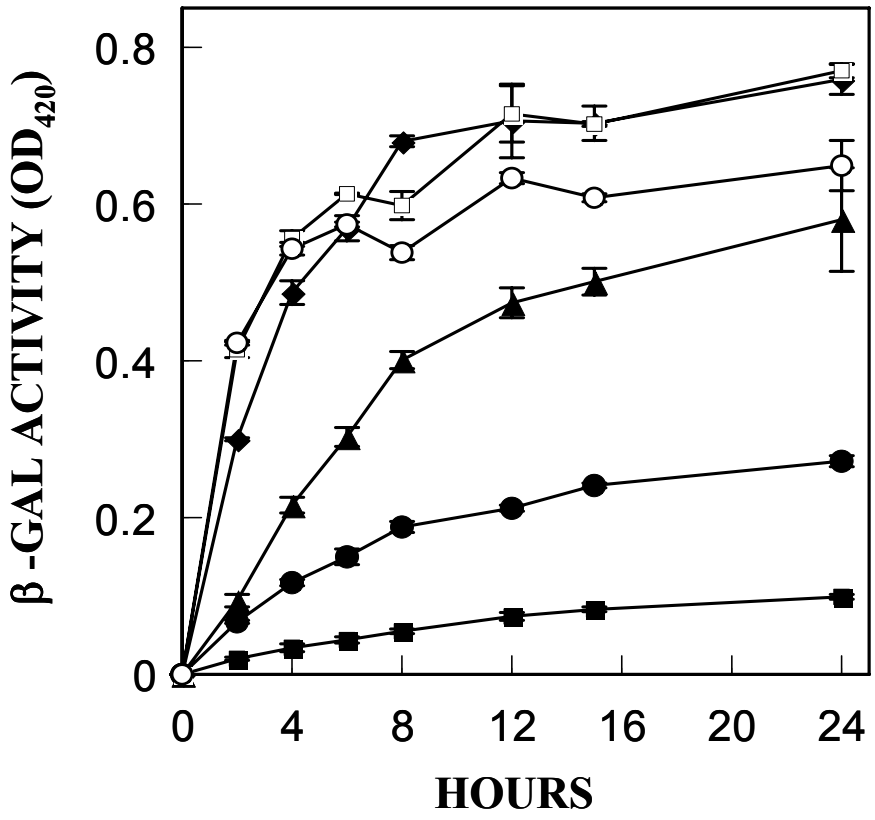

Figure 3.14 CSC and PB increase the rate of transduction. Retrovirus stocks (α-SGC-*LacZ*) were brought to 8 μg/ml PB (■), 80 μg/ml CSC and 88 μg/ml PB (●), 160 μg/ml CSC and 168 μg/ml PB (▲), 320 μg/ml CSC and 328 μg/ml PB (◆), 640 μg/ml CSC and 648 μg/ml PB (□), and 1000 μg/ml CSC and 1008 μg/ml PB (○), and then added to confluent cultures of NIH-3T3 cells. At various times, the virus was removed and replaced with fresh medium, and incubation continued at 37°C. Two days after the start of transduction, the level of gene transfer was quantified using the β-gal assay. Each point shows the mean ± standard deviation of three replicates.

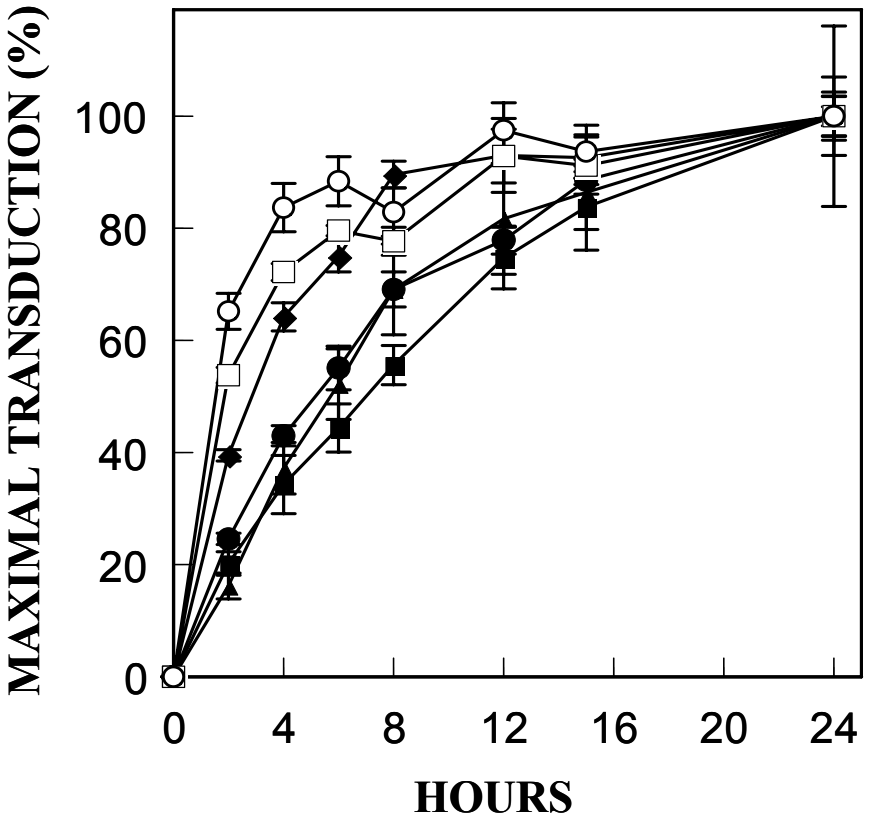

Figure 3.15 Time versus percent maximal infection for each of the polymer doses. Data from Figure 3.14.

the start of transduction. We found that the level of gene transfer was linearly proportional ($0.86 \leq r^2 \leq 0.99$) to the number of active particles delivered per cell (Figure 3.16, Table 3.2). The slopes of these linear correlations are a measure of the probability that an active virus, once it has reached the cell surface, will successfully complete post-binding steps of infection and transduce the cell. No statistically significant differences in the slopes of the lines were observed ($p < 0.05$), which indicates that an active virus that is associated with a polymer complex has the same probability of successfully transducing a cell once it has reached the cell surface as an active virus that is not associated with a polymer complex.

Other methods have been developed that increase the efficiency of gene transfer by increasing the rate that viruses are delivered to cells. For example, centrifugation of virus stocks onto cells (i.e., spinoculation) increases the efficiency of gene transfer. We wondered if gene transfer could be further improved if spinoculation were used, in combination with the formation of virus-polymer complexes, to transduce cells. Stocks of α-SGC-*lacZ* virus were brought to a range of concentrations of CSC and PB, added to cells, and then centrifuged (2000g) for 90 min. As a control, parallel cultures of cells were incubated with virus-polymer complexes but were not centrifuged. Virus was either left on the cells until they reached confluence, or removed and replaced with fresh medium 90 minutes after the start of transduction. The highest levels of gene transfer were achieved when virus-polymer complexes, formed using 320 μg/mL of PB and CSC, were incubated with cells for 48 hours without centrifugation (Figure 3.17), or when virus-polymer complexes, formed using 80 μg/mL of PB and CSC, were centrifuged onto the cells for 90 minutes, then immediately removed and replaced with fresh medium (Figure 3.18). Interestingly, when spinoculation was used, gene transfer declined if the dose of polymers exceeded 80 μg/mL and when the virus-polymer complexes were left

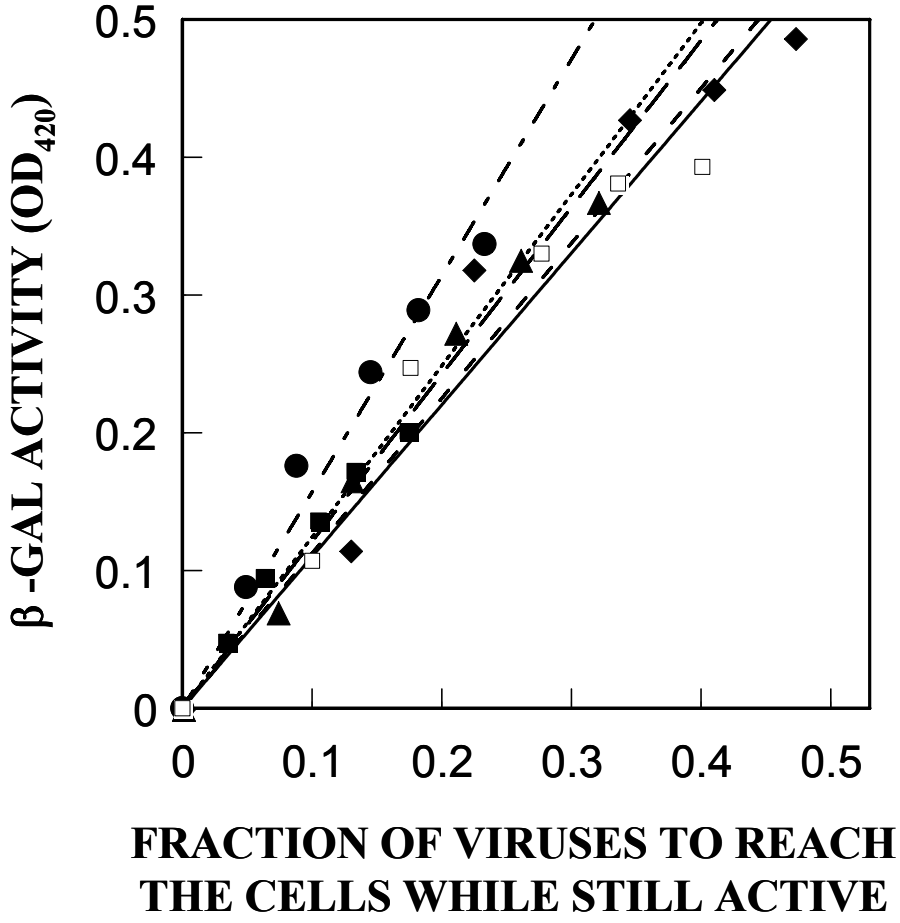

Figure 3.16 Complexation of virus with polymers does not affect the efficiency with which they complete post-binding steps of transduction. Retrovirus stocks (α-SGC-*LacZ*) were brought to 8 μg/ml PB (■, ·····), 80 μg/ml CSC and 88 μg/ml PB (●, — · —), 160 μg/ml CSC and 168 μg/ml PB (▲, — —), 320 μg/ml CSC and 328 μg/ml PB (◆, – – –), and 640 μg/ml CSC and 648 μg/ml PB (□, ——), and then added to cultures of NIH-3T3 cells. At various times, the supernatant was removed and frozen for later analysis, then replaced with fresh medium and incubation continued at 37°C. Two days after the start of transduction, the level of gene transfer was quantified using the β-gal assay. Samples of supernatant were thawed and analyzed for virus concentration using the p30 ELISA. The fraction of the total number of active viruses that were present at the start of transduction that reached the cells was estimated using a mathematical model of virus sedimentation and decay (see Materials and Methods). The level of gene transfer was plotted versus the fraction of active viruses delivered to the cells. Each point shows the mean ± standard deviation of three replicates. Lines, determined by the method of least squares, are the best-fit slopes of the data, which are proportional to the efficiency of post-binding steps of transduction.

Table 3.2 Polymers do not affect the efficiency of post-binding steps of transduction

CSC / PB concentration (μg/ml)	Best-fit slope[a] relative to 8 μg/ml PB	r^2
0 / 8	1.00 ± 0.00	0.99
80 / 88	1.30 ± 0.31	0.93
160 / 168	1.22 ± 0.53	0.86
320 / 328	1.19 ± 0.65	0.95
640 / 648	1.18 ± 0.46	0.94
1000 / 1008	0.93 ± 0.42	0.92

[a] Best-fit slopes were assumed to be proportional to the efficiency of post-binding steps of transduction.

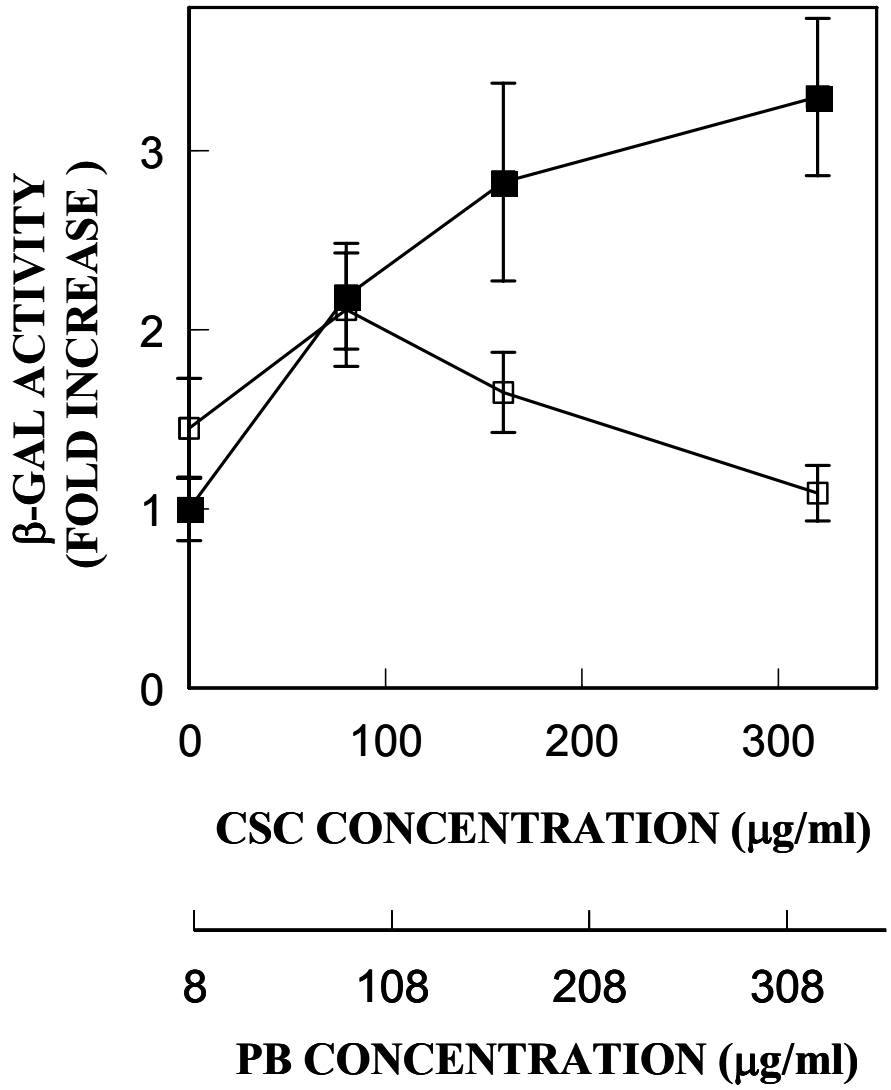

Figure 3.17 When the virus is left on the cells until they reach confluence, the highest transduction is achieved without spinoculation. Retrovirus stocks (α-SGC-*LacZ*) were brought to 0 to 320 mg/mL CSC and a weight concentration of PB that was 8 mg/mL more than that of CSC, added to cultures of NIH 3T3 fibroblasts, then the cells centrifuged (□) for 90 min at 2000g, or not centrifuged (■). The virus-laden supernatant was not replaced for the duration of the experiment. Cells were incubated for 2 days until confluent, and then the efficiency of gene transfer quantified using the β-gal assay. Results were reported as fold-enhancement with respect to the level of gene transfer achieved using the conventional transduction method in which virus and PB (8 μg/mL) only were used to transduce cells, without centrifugation.

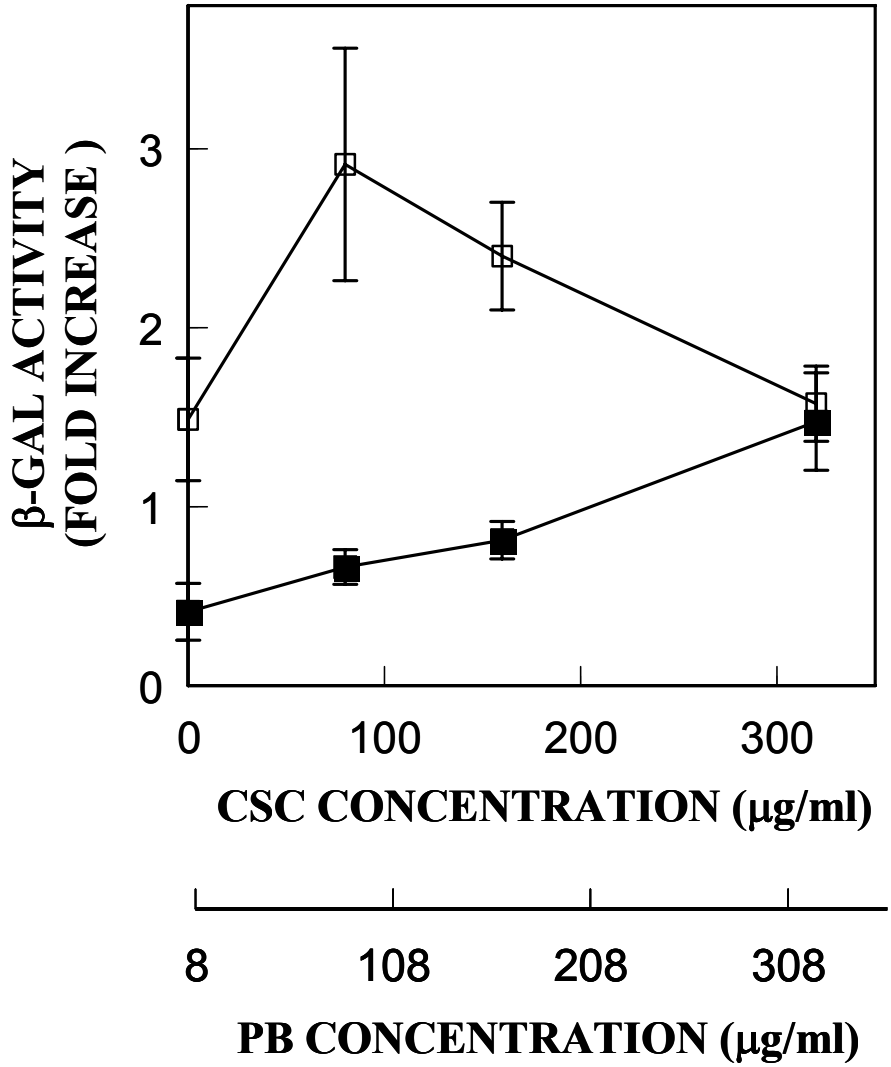

Figure 3.18 When the virus stock is removed from the cells shortly after the start of transduction, mixtures of CSC and PB increase the efficiency of gene transfer by spinoculation. Retrovirus stocks (α-SGC-*LacZ*) were brought to 0 to 320 mg/mL CSC and a weight concentration of PB that was 8 mg/mL more than that of CSC, added to cultures of NIH 3T3 fibroblasts, then the cells centrifuged (□) for 90 min at 2000g, or not centrifuged (■). The virus-laden supernatant was replaced with fresh medium 90 minutes after the start of transduction (immediately after centrifugation). Cells were incubated for 2 days until confluent, and then the efficiency of gene transfer quantified using the β-gal assay. Results were reported as fold-enhancement with respect to the level of gene transfer achieved using the conventional transduction method in which virus and PB (8 μg/mL) only were used to transduce cells, without centrifugation.

on the cells for the duration of the experiment. These results show that high levels of gene transfer can be realized through the use of virus-polymer complexes without the use of spinoculation, but that spinoculation can reduce the amount of polymers that need to be used, and the length of time cells need to be exposed to virus.

3.5 Discussion

Charged polymers are known to significantly affect retrovirus transduction. In general, it appears that cationic polymers enhance transduction by increasing virus binding whereas anionic polymers inhibit transduction by decreasing virus binding [14, 22]. Although the mechanism by which charged polymers affect virus binding has not been thoroughly investigated, it is generally thought that cationic polymers reduce the electrostatic repulsion between the negatively charged viruses and cells, and that anionic polymers have the opposite effect. In addition, some anionic polymers (e.g., heparin and heparan sulfate) appear to act by binding to the heparan-binding domain of fibronectin, a region of fibronectin to which retroviruses bind [22], or to domains on the virus that would otherwise bind to cell-associated glycosaminoglycans [19]. We were surprised then to find that retrovirus transduction is more efficient when both anionic and cationic polymers are added to virus stocks than when only cationic polymers are used. Since our observation seemed to conflict with the current model of charged polymer – virus interactions, we decided to determine the mechanism by which transduction was enhanced.

Using quantitative assays of retrovirus transduction and binding, we found that the addition of oppositely charged polymers (PB and CSC) to virus stocks enhanced gene transfer by increasing the flux of active viruses to the cells. Virus-polymer complexes formed that did not reduce the stability of the viruses, yet were large enough to sediment, delivering the viruses to the cells more rapidly than by simple diffusion. As

the concentration of the polymers increased, the size of the complexes increased, leading to an increase in the rate of sedimentation. This was expected because the velocity with which particles sediment (v_{sed}) increases with the square of their radius (R) and is a function of the difference between the density of the particle and the density of the medium $(\rho_s - \rho)$, gravitational constant (g), and the viscosity of the medium (η) [31, 32]:

$$v_{sed} = 2R^2(\rho_s - \rho)g/9\eta \quad (10)$$

Viruses that were associated with these larger complexes sedimented faster, were more likely to bind to cells before they lost their bioactivity, and led to higher levels of gene transfer.

When high concentrations of only one polymer were used, visible complexes were not formed and gene transfer was not enhanced, but decreased to undetectable levels. This decrease is consistent with previous data showing that high concentrations of PB in the absence of an anionic polymer are cytotoxic (refer to Chapter 2) [24], and that high concentrations of CSC in the presence of low concentrations of PB inhibit gene transfer [21].

We tested the ability of the polymers to enhance transduction using a number of different cell types and viruses. Transduction was enhanced in all of our experiments but to a degree that varied depending on the type of cells and viruses used. For example, transduction was enhanced the most with cells that were difficult to transduce using the conventional protocol in which only one polymer, Polybrene, was used. This is probably because the rate of virus binding when both polymers were used was a function only of the sedimentation rates of the virus-polymer complexes that formed and

was therefore the same regardless of the cell type that was transduced. In contrast, the rate of virus binding when only Polybrene was used may have been a function of the properties of the cells that were transduced, such as how avidly Polybrene was able to bind to them. This rate may have been lower in cell types that were relatively refractory to Polybrene-mediated transduction, such as HeLa cells, than in cell types that were more susceptible to Polybrene-mediated transduction, such as NIH-3T3 cells.

The properties of the virus stocks also appeared to influence the degree to which the complexes enhanced transduction. Transduction of HeLa cells was enhanced more than 30-fold when viruses produced by murine fibroblasts (Ψ-CRIP) were used, but only 2–fold when viruses produced by TELCeB6-A cells were used. Similarly, transduction of NIH 3T3 cells was enhanced nearly 8-fold when amphotropic retroviruses were used, but only 3-fold when amphotropic lentiviruses were used. Lentivirus transduction may have been enhanced to a lesser extent than retrovirus transduction because lentiviruses lose their activity more slowly than do retroviruses (data not shown). In addition, virus stocks produced by TELCeB6-A cells contained high levels of inhibitory activity which may explain why polymer-complexation enhanced their efficiency of gene transfer to a lesser extent than when other virus stocks were used that contained significantly less inhibitory activity (refer to Chapter 5).

We wondered if viruses that were associated with complexes were hindered in their ability to transduce cells once they bound to the cell surface. Using a simple mathematical model and experimental measurements of the rates of virus sedimentation and decay, we estimated, as a function of polymer concentration, the rate that active virus particles reached the surfaces of the cells and the efficiency with which they completed post-binding steps of transduction. Surprisingly, we found that association of the viruses with complexes did not significantly hinder their ability to complete post-binding steps of transduction. Perhaps the viruses do not remain associated with the

complexes once they reach the cells, either because the complexes are degraded by cellular enzymes, or because the complexes are in a continuous state of disaggregation and reaggregation that provides the viruses with transient opportunities to escape from the complexes. Also, given that most of the viruses appear to be located on the outer surface of the complexes, it is possible that the complexes do not block their access to their cellular receptors.

The formation and sedimentation of virus-polymer complexes is most likely a form of flocculation, the destabilization of a suspension of colloids by the addition of a chemical agent known as a flocculant [33]. Charged polymers have been used as flocculants in a number of industrial settings including in the processing of minerals and pulp, in the purification of proteins and wastewater, and as a method to control the surface properties and behavior of biopharmaceuticals [34]. Highly charged, relatively low molecular weight polymers such as those used in this study typically induce flocculation by binding to oppositely charged sites on the surfaces of the colloids, forming an “electrostatic patch” that then binds to oppositely charged patches on adjacent particles [33]. In principle, virus-polymer complexes can be formed using a single type of cationic polymer. For example, Zhang et al formed lentivirus-poly-L-lysine complexes as a means to more efficiently concentrate lentiviruses by centrifugation [35]. We have previously found, however, that if two oppositely charged polymers (e.g., CSC and PB) are used to form the complexes, it is possible to achieve high levels of gene transfer by applying the virus-polymer complexes directly to the cells, without the need for centrifugation [24]. We suspect that the negatively charged polymer (CSC) helps to protect the cells from what would otherwise be a cytotoxic dose of the cationic polymer (PB). Regardless of the precise mechanism by which charged polymers induce viruses to flocculate and sediment onto cells, our studies show that the transport of retroviruses to cells is a major rate-limiting step of transduction. This observation is consistent with a

number of previous studies, including one that showed that virus binding is diffusion limited and occurs on a much longer time scale than does virus decay when conventional transduction protocols are used [7].

The formation of retrovirus-polymer complexes may have a number of important applications. Similar to other methods that rapidly deliver retroviruses to cells, they can be used to significantly improve the efficiency of *ex vivo* gene transfer [36-39]. Virus-polymer complexes are not cytotoxic, are straightforward and inexpensive to form, can be formed with retroviruses and lentiviruses regardless of the type of viral envelope protein they are pseudotyped with, and can be used to rapidly transduce cells without the need for specialized equipment such as centrifuges, flow-through tissue culture devices, or magnetic beads and magnets. As shown in our previous study, retrovirus-polymer complexes can also be used to concentrate and purify retrovirus stocks, resulting in substantial enhancement of transduction [24]. This concentration method is a promising alternative to other methods that are time-consuming and can only accommodate small volumes, such as ultracentrifugation. In addition, our method permits resuspending viruses in culture medium that is optimal for the proliferation of the intended target cell type and may therefore further increase gene transfer. Recently, we have successfully enhanced gene transfer to murine bone marrow progenitor cells by concentrating stocks of ecotropic retrovirus using PB and CSC. We found that a 10-fold concentration resulted in a 4-fold enhancement of transduction (personal communication, Spencer, HT, Emory University, 2004).

In summary, we have shown that the combined addition of oppositely charged polymers to retrovirus stocks leads to the formation of virus-polymer complexes that can be used to overcome some of the major rate-limiting steps of retroviral-mediated gene transfer. Virus-polymer complexes sediment, increasing the number of active viruses that are delivered to the cells, and are easily concentrated and purified by low speed

centrifugation and resuspension in fresh medium. Future studies are needed to determine the properties of the viruses and polymers that are necessary for complex formation, and the mechanism by which viruses are able to transduce cells as part of a high-molecular-weight complex.

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CHAPTER 4

FLOCCULATION OF RETROVIRUSES WITH VARIOUS COMBINATIONS OF OPPOSITELY CHARGED POLYMERS OR WITH A SINGLE SPECIES OF CATIONIC POLYMER INCREASES THE EFFICIENCY OF GENE TRANSFER

4.1 Abstract

We have shown in Chapters 2 and 3 that the combined addition of high and equal weight concentrations (80 to 1000 μg/mL) of the cationic polymer Polybrene (PB) and the anionic polymer chondroitin sulfate C (CSC) to stocks of retroviruses results in the formation of high molecular weight complexes. These complexes selectively incorporate virus particles and enhance the efficiency of transduction by increasing the rate of binding of viruses to cells. In addition, these complexes can be rapidly pelleted by centrifugation in a table top centrifuge. Resuspension of the pellet to smaller volumes further enhances gene transfer. While flocculation of viruses with combinations of PB and CSC appears to be an effective means for enhancing transduction, the possibility of inducing flocculation with other polymers has not been investigated. In this study we used five cationic polymers and seven anionic polymers to assess the efficiency of dual flocculation of viruses with oppositely charged polymers and its effect on transduction. Viruses aggregated into complexes regardless of the combination we tried, and enhanced gene transfer between 2.5 and 5.5-fold. In each case, gene transfer was maximal when the number of positive charges and the number of negative charges added to the stock was about equal.

While investigating the possibility of dual flocculation with various polymers, we found that it is possible to concentrate viruses by flocculation with a single species of

cationic polymer. Viruses concentrated after single flocculation significantly enhanced gene transfer, but to a lesser extent than viruses concentrated after dual flocculation. The difference in enhancement of transduction between these two concentration methods was due to the fact that viruses concentrated after dual flocculation remained as part of polymer complexes and were rapidly transported to the cells. In contrast, viruses concentrated after single flocculation appeared to disaggregate from the complexes and were transported mainly by diffusion. We concluded that concentration by single flocculation may be a useful alternative method for concentrating and purifying virus for applications in which high concentrations of polymers are undesirable or when it is not necessary for viruses to rapidly sediment onto the target cells.

4.2 Introduction

The presence of charged polymers in stocks of retroviruses can significantly alter the efficiency of transduction. Cationic polymers have been shown to enhance gene transfer and are commonly added to stocks of retroviruses during transduction [1-3]. While the mechanism of enhancement is not fully understood, some studies indicate that these polymers increase the rate of binding of virus particles to target cells [4, 5]. Most likely, positively charged polymers reduce the electrostatic repulsion between the negatively charged surfaces of viruses and cells [6]. Alternatively, they may induce aggregation of viruses into complexes, which are more susceptible to sedimentation than free virus particles [6]. In spite of their potential for enhancing retrovirus transduction, the use of these polymers has been limited to low concentrations (2 to 20 μg/ml in most cases), because they are cytotoxic at higher doses [1-3] .

Anionic polymers, on the other hand, have been shown to almost always inhibit retrovirus transduction [7]. The mechanism of inhibition is not well understood, but these

polymers appear to decrease the rate of binding of viruses to cells [8-11], probably by increasing the electrostatic repulsion between viruses and cells. Some of the anionic polymers present in virus stocks are a byproduct of the virus production system. They are secreted by packaging cells in the form of glycosaminoglycans and proteoglycans [7, 11]. However, not all these polymers inhibit transduction. For example, chondroitin sulfate proteoglycans and glycosaminoglycans appear to inhibit gene transfer only if they have a high degree of sulfation and a specific carbohydrate structure [11]. In addition to increasing repulsion between viruses and cells, glycosaminoglycans that do inhibit transduction appear to prevent non-specific binding of viruses to cells. This binding step can be mediated by interactions between the surface of the virus and glycosaminoglycans expressed on the surface of the cells [9]. Alternatively, binding may result from interactions between glycosaminoglycans on the surface of the viruses and heparan-binding domains of fibronectin molecules bound to the surfaces of cells or tissue culture plates [12]. Thus, free glycosaminoglycans are likely to compete with cell-associated glycosaminoglycans for virus binding or with virus-associated glycosaminoglycans for binding to the cells through fibronectin [9, 12]. Regardless of the mechanism of inhibition, removal of anionic polymers from virus stocks has been shown to improve transduction [13].

Interestingly, we found that addition of high concentrations of the glycosaminoglycan chondroitin sulfate C (CSC) to a retrovirus stock can substantially increase gene transfer when added in conjunction with a high and equal weight concentration of the cationic polymer Polybrene (PB) [14, 15]. Addition of PB and CSC to stocks of retroviruses results in the formation of retrovirus-polymer complexes that incorporate more than 80% of the viruses and less than 0.3% of the proteins originally present in the stock [14]. These complexes do not reduce the stability of the viruses, yet are large enough to sediment, delivering the viruses to the cells more rapidly than by

simple diffusion. Moreover, the complexes are not cytotoxic even though they contain high concentrations of cationic polymer. Rather, increasing doses of PB and CSC correlate with increasing levels of gene transfer (refer to Chapter 3) [15].

Viruses within polymer complexes not only benefit from an accelerated binding to cells, but they can also be rapidly and easily concentrated and purified. Centrifugation of the complexes for 5 min at 10000g, followed by resuspension of the pellet in a reduced volume of fresh cell culture medium has resulted in substantial enhancement of transduction (refer to Chapter 2) [14].

While association of virus particles with polyelectrolyte complexes of PB and CSC appears to be an effective means for enhancing the efficiency or retroviral-mediated gene transfer, the possibility of forming virus-polymer complexes with other charged polymers with the aim of improving transduction has not been investigated. The formation of complexes in the presence of PB and CSC is most likely a form of flocculation, the destabilization of a suspension of colloids by the addition of a chemical agent known as a flocculant [16]. Charged polymers have been used as flocculants in a number of industrial settings including in the processing of minerals and pulp, in the purification of proteins and wastewater, and as a method to control the surface properties and behavior of biopharmaceuticals [17-24]. Highly charged, relatively low molecular weight polymers such as those used in this study typically induce flocculation by binding to oppositely charged sites on the surfaces of the colloids, forming an “electrostatic patch” that then binds to oppositely charged patches on adjacent particles [16]. In principle, and in analogy with other viruses, retrovirus-polymer complexes can be formed using a single species of cationic polymer (single flocculation) [25-27]. For example, Zhang et al formed lentivirus-poly-L-lysine complexes as a means to more efficiently concentrate lentiviruses by centrifugation [28]. In our system, flocculation with two oppositely charged polymers (dual flocculation), permits achieving high levels of

gene transfer by applying the virus-polymer complexes directly to the cells [15]. This suggests that the negatively charged polymer CSC protects the cells from what would otherwise be a cytotoxic dose of the cationic polymer PB.

In the present study, we examined the possibility of inducing flocculation of viruses and enhancing gene transfer using different combinations of oppositely charged polymers. To examine this possibility, we hypothesized that virus flocculation was driven by interactions with a cationic polymer, and that an anionic polymer was required during transduction to neutralize the toxicity of the cationic polymer.

4.3 Materials and methods

Chemicals and antibodies. Chondroitin sulfate A sodium salt from bovine trachea (CSA), chondroitin sulfate B sodium salt (CSB), chondroitin 6-sulfate sodium salt from shark cartilage (CSC), dextran sulfate sodium salt Leuconostoc spp. (DS), Heparin sodium salt from porcine intestinal mucosa (HEP), de-N-sulfated heparin sodium salt (desHEP), hyaluronic acid potassium salt from human umbilical cord (HA), hexadimethrine bromide (Polybrene, PB), Poly-L-lysine hydrobromide (PLL), protamine sulfate salt Salmon (PS), gluteraldehyde, , o-Nitrophenyl β-D-galactopyranose (ONPG) and Igepal CA-630 were from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide 30%, and Polyoxyethylene 20-Sorbitan Monolaurate (Tween 20) were from Fisher Scientific (Fair Lawn, NJ). Non-fat dry milk (blotting grade) was from Bio-Rad Laboratories (Hercules, CA). o-Phenylenediamine Dihydrochloride (OPD) was from Pierce (Rockford, IL). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Denville Scientific, Inc. (Metuchen, NJ). Mouse anti-p30 antibodies were purified from the supernatant of the CRL-1219 hybridoma cell line (ATCC, Rockville, MD) following standard procedures [29]. The goat polyclonal anti-p30 antibody (78S221) was

from Quality Biotech (Camden, NJ). The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories (South San Francisco, CA).

Cell culture. NIH 3T3 mouse fibroblasts and the amphotropic packaging cell line Ψ-CRIP producing the α-SGC-*LacZ* virus [30], were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% bovine calf serum (Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Hyclone Labs Inc.) and 110 μg/mL of sodium pyruvate (Hyclone Labs Inc.) (DMEM/BCS).

Production of retroviruses. To generate retrovirus stocks, virus-producing cells were grown to confluence in T175 tissue culture flasks, and then incubated for 24 h with 35 mL of DMEM/BCS. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use.

ELISA for p30. We used an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of virus capsid protein (p30). ELISA plates (Nunc immuno Maxisorp 96-well plates, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to block non-specific binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 was sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer, and incubated for 1 h at 37°C. The horseradish peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed for 5 min using

hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Virus flocculation, precipitation and resuspension. We compared two methods to flocculate virus particles: single flocculation and dual flocculation. For single flocculation, virus stocks were brought to various concentrations of PB and incubated at 37°C for 20 min to allow aggregation of viruses and molecules of PB. The aggregates were concentrated in an invisible pellet by centrifugation for 5 min at 10000g. The supernatant was decanted and the pellet resuspended in fresh medium by pipetting up and down. For dual flocculation, virus stocks were brought to various concentrations of a cationic polymer and to various concentrations of an anionic polymer (refer to Table 4.1 for a list of cationic and anionic polymers), then incubated at 37°C for 20 min to allow aggregation of polymers and viruses into visible polyelectrolyte complexes. The complexes were pelleted by centrifugation for 5 min at 10000g. The supernatant was decanted and the pellet resuspended in fresh medium by pipetting up and down.

Cell viability assay. Ten microliters per well of MTT solution (100 mg of MTT in 1 mL of PBS) were added per well to cells in a 96-well plate. The plate was incubated for 4 hours at 37°C, then 150 μL of 10% SDS were added per well and the plate incubated overnight. The optical density at 570 nm was measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells

without cells were subtracted as background. Values for each point are the average of triplicate wells.

β-galactosidase (β-gal) assay. To test the effect of polymers on transduction, five thousand NIH 3T3 murine fibroblasts in 100 μL of medium were plated per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, MA). The next day (19 to 26 h later), the medium was removed and 100 μL of the virus stocks were added to each well. Two days after transduction, the medium was removed and the cells washed once with 100 μL of phosphate-buffered saline (PBS) containing 1mM $MgCl_2$. After removal of the wash solution, 50 μL of lysis buffer (PBS with 1mM $MgCl_2$ and 0.5% Igepal) were added to each well, and the plate incubated at 37°C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG warmed to 37°C were added to each well, and the plate incubated at 37°C for 5 to 15 min until a visible yellow color was obtained. The reactions were halted by the addition of 20 μL per well of stop buffer (1M Na_2CO_3). The optical density at 420 nm (OD_{420}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA) and the non-specific background at 650 nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.

Ratio and concentration of polymers that maximize the efficiency of transduction. Virus stocks were brought to low (80 μg/mL) or high (640 μg/mL) concentrations of PB and to various concentrations to one of the anionic polymers listed in Table 4.1. Alternatively, virus stocks were brought to low (80 μg/mL) or high (640 μg/mL) concentrations of CSC and to various concentrations to one of the cationic polymers listed in Table 4.1. These stocks were added to NIH 3T3 fibroblasts that were plated the previous day at 5000 cells per well in a 96-well dish. Two days later, the

efficiency of transduction was quantified using the β-galactosidase assay. In each case, the ratio of anionic to cationic polymer that resulted in maximal transduction was determined (Table 4.2). To examine the concentration of polymers that maximized the gene transfer, various concentrations of the cationic polymer and the anionic polymer were added to virus stocks at the ratio previously determined. These stocks were used to transduce NIH 3T3 cells and the efficiency of gene transfer was quantified two days later. In each case, transduction increased with increasing concentrations of the polymers, reached a plateau level and eventually decreased with increasing concentrations of polymers. The concentrations of polymers that led to the highest transduction efficiencies were reported in Table 4.2.

Ratio of positive to negative charges added to the virus stock. Based on the chemical structure of each polymer, the molecular weight of the monomer (MW) and the number of positive charges (P) or negative charges (N) per monomer at neutral pH were determined. The total number of positive charges (TP) or negative charges (TN) corresponding to a mass m of polymer added to a stock was calculated using the following equations:

$$TP = \frac{P * m * Nav}{MW}$$

$$TN = \frac{N * m * Nav}{MW}$$

where Nav is the Avogadro's number. The ratio of positive charges to negative charges added to the stock was determined by dividing TP by TN.

4.4 Results

Flocculation of viruses with a single charged polymer

To investigate the possibility that viruses aggregate with polyelectrolyte complexes by interacting primarily with a positively charged group, we examined if aggregation of viruses was possible when only a cationic or anionic polymer was added to the stock. For that purpose, we used the criterion that while free viruses are subject to diffusion limitations, viruses within aggregates sediment, thereby reaching the target cells faster than free viruses (refer to Chapter 3) [15]. We brought stocks of retroviruses to 320 μg/mL of PB, 320 μg/mL of CSC, a combination of 320 μg/mL of PB and 320 μg/mL of CSC, or, as a control, 8 μg/mL of PB. We added these stocks to layers of NIH 3T3 cells and, at various times, measured the concentration of viral capsid protein (p30) remaining in solution. When 320 μg/mL of CSC were used, the rate of disappearance of viruses from solution was similar to that in the control. However, when 320 μg/mL of PB were used, the rate of disappearance of viruses from solution was higher than that of the control, but lower than that in the presence of both polymers (Figure 4.1). This suggested that PB, but not CSC, could induce flocculation of retroviruses.

To directly evaluate the possibility that viruses flocculate in the presence of PB, we brought virus stocks to various concentrations of PB. We incubated the stocks for 20 min to allow aggregation of viruses, then centrifuged the stocks and decanted the supernatants. We did not observe pellets at the bottom of each tube, but we assumed the pellets were invisible and resuspended them to the original volume in fresh medium. As a control, we also pelleted virus stocks in the presence of 80 μg/mL of PB and 80 μg/mL of CSC, and, as expected, obtained a visible pellet (refer to Chapter 2). To examine if aggregates of virus particles were precipitated, we measured the concentration of p30 in the original virus stocks, the supernatants and the pellets. In the

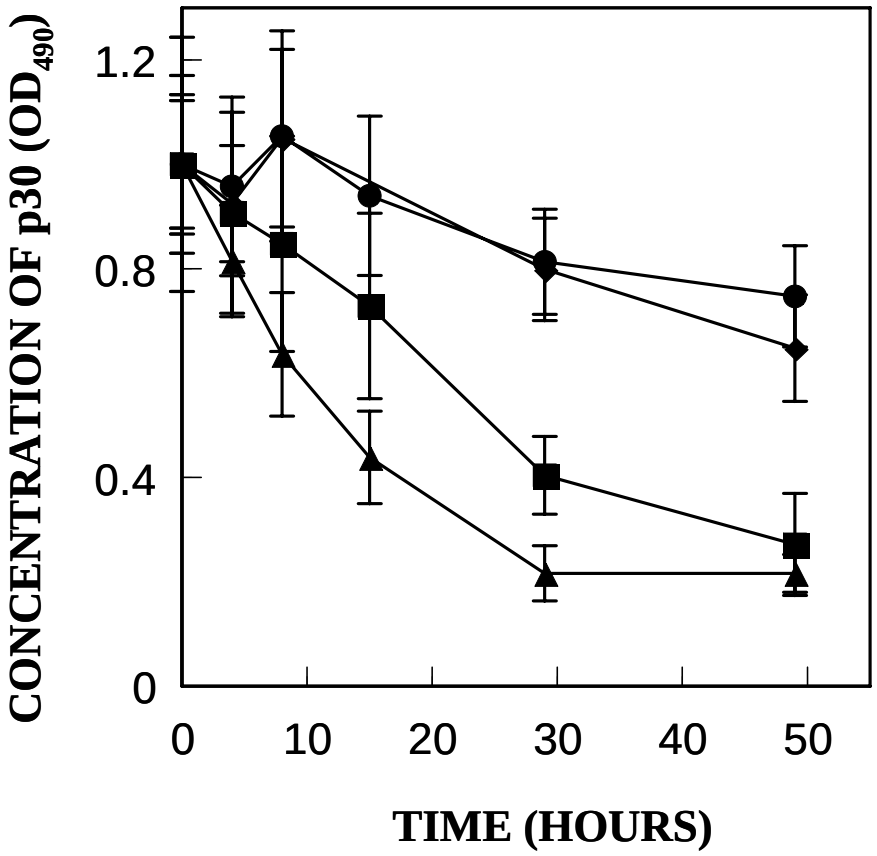

Figure 4.1 High concentrations of PB, but not CSC, increase the rate of binding of viruses to cells. Virus stocks were brought to 320 μg/mL of PB (■), 320 μg/mL of CSC (●), to a combination of 320 μg/mL of PB and 320 μg/mL of CSC (▲), or to 8 μg/mL of PB (◆), then added to NIH 3T3 fibroblasts. At various times, the supernatant was collected and the concentration of virus capsid protein (p30) remaining in the supernatant was quantified by ELISA. Each point represents the mean ± the standard deviation of three samples.

presence of only PB, the percentage of viral proteins in the pellet increased with increasing concentrations of the polymer (Figure 4.2). When 320 μg/mL of PB were used, the recovery of viruses was similar to that obtained with 80 μg/mL of both PB and CSC. These results indicated that PB could, in fact, flocculate viruses.

Given that the pellets obtained after flocculation with only PB were not visible, we suspected that only a small fraction of the PB added to the stock was incorporated into the complexes. To study this possibility, we took into account our previous finding that high concentrations of PB are toxic to target cells (refer to Chapter2) [14]. Therefore, we hypothesized that upon centrifugation of the aggregates, the pellet would only concentrate a small fraction of PB and not be toxic. To test this hypothesis, we brought virus stocks to various concentrations of PB. After a 20-min incubation, we pelleted the stocks, collected the supernatants and resuspended the pellets to their original volume in fresh medium. As a control, we also pelleted virus stocks containing 80 μg/mL of PB and 80 μg/mL of CSC. We added the virus stocks, the supernatants and the resuspended pellets to layers of cells and, two days later, measured cell viability. The supernatants containing free PB were as toxic as the original stocks, while the pellets were not toxic (Figure 4.3). This supported our hypothesis that only a small fraction of PB aggregated with virus particles.

Taken together, our results indicated that viruses could flocculate by interacting with a positively charged polymer, but not with a negatively charged polymer. This suggested that viruses adsorb mainly to positively charged patches of polyelectrolyte complexes during dual flocculation.

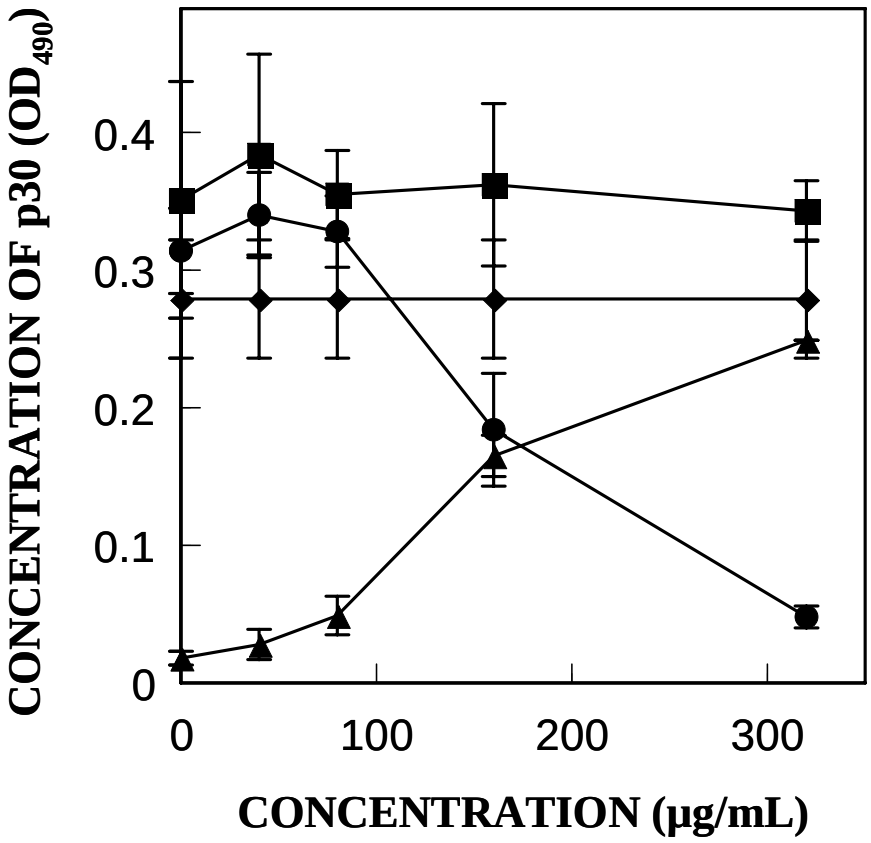

Figure 4.2 PB induces aggregation of virus particles. Virus stocks were brought to various concentrations of PB, incubated at 37°C for 20 min and centrifuged at 10000g for 5 min. The supernatant was decanted and the pellet resuspended to the original volume in fresh medium. As a control, virus stocks were brought to 80 μg/mL of PB and 80 μg/mL of CSC, then pelleted in a similar manner. The concentration of p30 in virus stocks (■), the decanted supernatants (●), the resuspended pellets (▲) and the control pellet (◆) were quantified by ELISA. (Note that the control pellet does not contain varying concentrations of PB). Each point represents the mean ± the standard deviation of three samples.

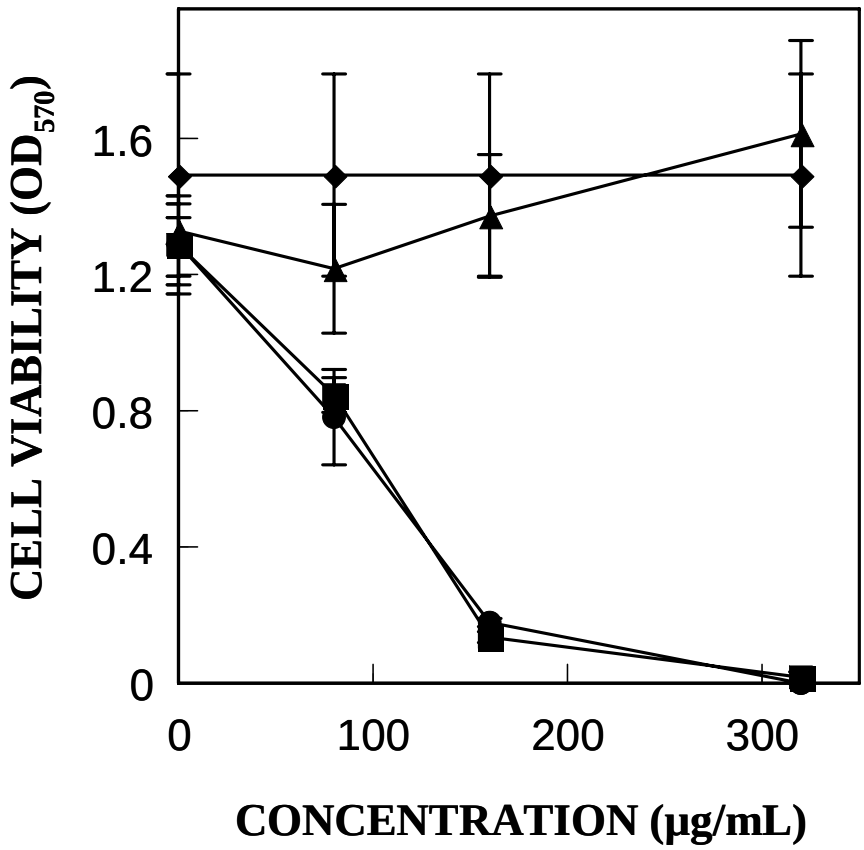

Figure 4.3 Aggregates of viruses and PB are not toxic. Virus stocks were brought to various concentrations of PB, incubated at 37°C for 20 min and centrifuged at 10000g for 5 min. The supernatant was decanted and the pellet resuspended to the original volume in fresh medium. As a control, virus stocks were brought to 80 μg/mL of PB and 80 μg/mL of CSC, then pelleted in a similar manner. The virus stocks (■), the decanted supernatants (●), the resuspended pellets (▲) and the control pellet (◆) were added to NIH 3T3 cells and two days later, cell viability was measured. (Note that the control pellet does not contain varying concentrations of PB). Each point represents the mean ± the standard deviation of three samples.

Dual flocculation of viruses with oppositely charged polymers

We have previously shown that while high doses of PB are cytotoxic, combinations of equal weight concentrations of PB and CSC are not (refer to Chapter 2) [14]. Based on this finding, we hypothesized that CSC, the anionic polymer, mitigated the toxicity of PB, the cationic polymer by neutralizing the excess of positive charge. To test this hypothesis, we first assessed the extent to which anionic polymers other than CSC could mitigate the toxicity of PB. We incubated cells for two days with virus stocks containing 640 μg/mL of PB and a range of concentrations of one of the anionic polymers listed in Table 4.1: chondroitin sulfate A (CSA), chondroitin sulfate (CSB), CSC, heparin (HEP), de-N-sulfated heparin (desHEP), dextran sulfate (DS) or hyaluronic acid (HA). In all the cases, high concentrations of the anionic polymer mitigated the toxic effect of PB (Figures 4.4 - 4.5). We also examined the possibility that CSC could mitigate the toxicity of other cationic polymers. We incubated cells with virus stocks containing 640 μg/mL of CSC and various concentrations of one of the cationic polymers listed in Table 4.1: PB, poly-l-lysine of low molecular weight (PLL (low)), poly-l-lysine of medium molecular weight (PLL (med)) or poly-l-lysine of high molecular weight (PLL (high)). As expected, an excess of any cationic polymer decreased cell viability (Figure 4.6). These results suggested that the anionic polymer reduced the toxicity of the cationic polymer by neutralizing the excess of positive charge in the medium.

Based on these findings and the observation that viruses interacted mainly with PB, we hypothesized that viruses would aggregate with complexes formed by PB and any of the anionic polymers listed in Table 4.1, and that viruses in these complexes would transduce cells more efficiently than free viruses. To test this hypothesis, we transduced cells with virus stocks containing 640 μg/mL of PB and a range of concentrations of each of the anionic polymers listed in Table 4.1. In each case, gene

Table 4.1 List of polymers

Polymer	Abbreviation used	Anionic/ cationic	Charged groups	Molecular weight (kDa) [a]	Linear/branched
Polybrene	PB	Cationic	amino	5-10	Linear
Protamine sulfate	PS	Cationic	amino	5	Linear
Poly-L-lysine (low MW)	PLL (low)	Cationic	amino	4-15	Linear
Poly-L-lysine (medium MW)	PLL (med)	Cationic	amino	30-70	Linear
Poly-L-lysine (high MW)	PLL (high)	Cationic	amino	>300	Linear
Chondroitin sulfate A	CSA	Anionic	sulfate, carboxyl	20-30	Linear
Chondroitin sulfate B	CSB	Anionic	sulfate, carboxyl	13	Linear
Chondroitin sulfate C	CSC	Anionic	sulfate, carboxyl	60	Linear
Heparin	HEP	Anionic	sulfate, carboxyl	16-17	Linear
De-N-sulfated heparin	desHEP	Anionic	sulfate, carboxyl	17-18	Linear, de-N-sulfated
Dextran sulfate	DS	Anionic	sulfate	500	Branched
Hyaluronic acid	HA	Anionic	carboxyl	750	Linear

a: Provided by Sigma-Aldrich technical support

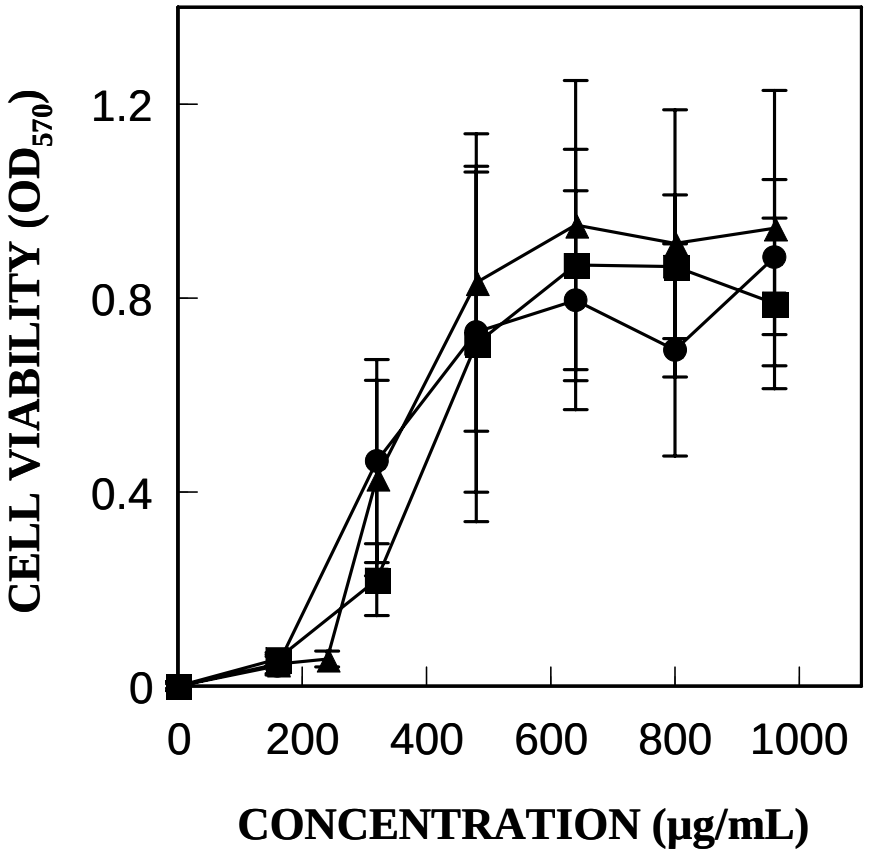

Figure 4.4 Anionic polymers mitigate the toxicity of cationic polymers. Virus stocks were brought to 640 μg/mL of PB and to various concentrations of CSA (■), CSB (●) or CSC (▲), then added to NIH 3T3 cells. Two days later cell viability was quantified. Each point represents the mean ± the standard deviation of three samples.

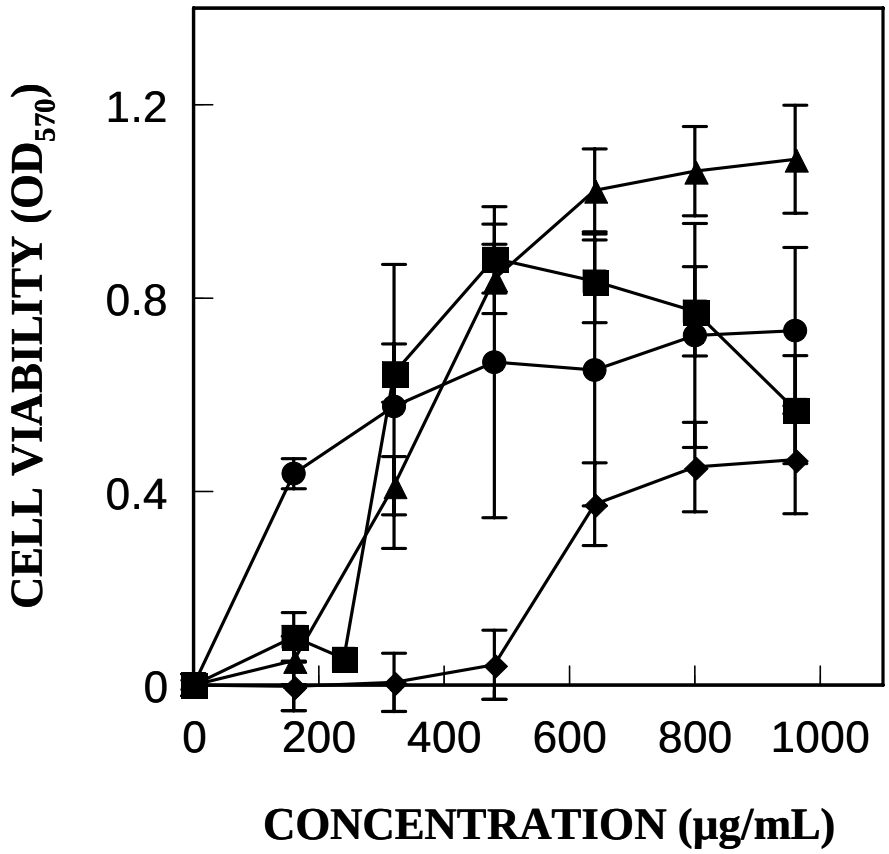

Figure 4.5 Anionic polymers mitigate the toxicity of cationic polymers (continue). Virus stocks were brought to 640 μg/mL of PB and to various concentrations of DS (■), HEP (●), desHEP (▲) or HA (◆), then added to NIH 3T3 cells. Two days later, cell viability was quantified. Each point represents the mean ± the standard deviation of three samples.

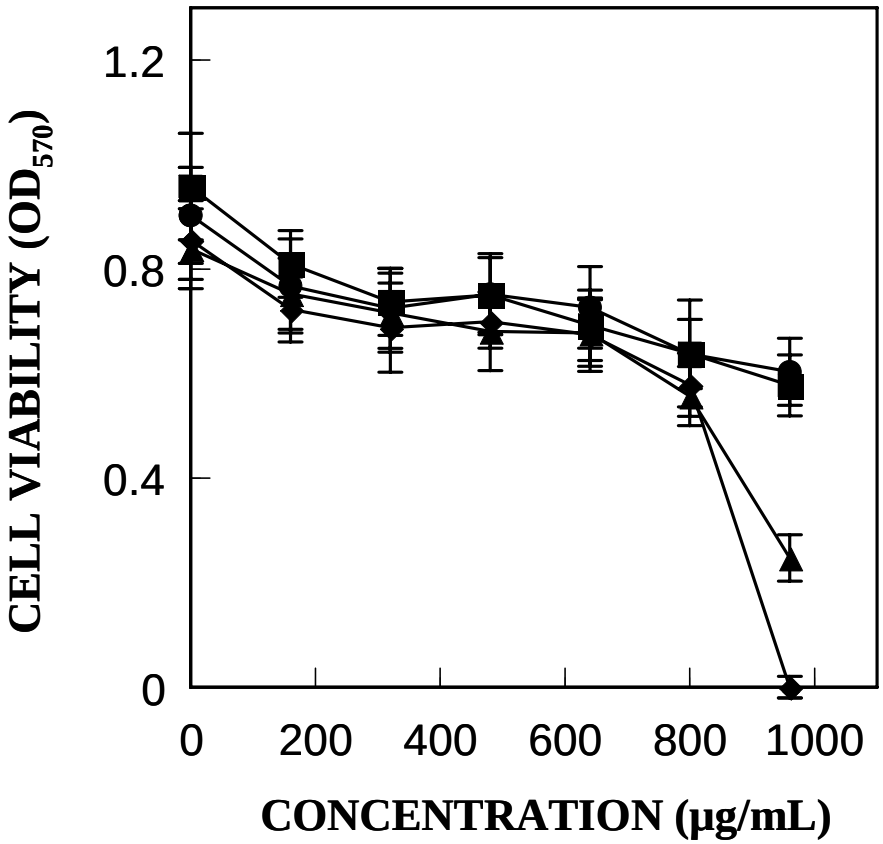

Figure 4.6 Anionic polymers mitigate the toxicity of cationic polymers (continue). Virus stocks were brought to 640 μg/mL of CSC and to various concentrations of PB (■), PLL (low) (●), PLL (med) (▲) or PLL (high) (◆), then added to NIH 3T3 cells. Two days later, cell viability was quantified. Each point represents the mean ± the standard deviation of three samples.

transfer increased with increasing concentrations of the anionic polymer, reached a maximum and then decreased with increasing concentrations of the anionic polymer (Figures 4.7-4.8). This indicated that aggregation of viruses within polyelectrolyte complexes was possible in the presence of anionic polymers with different characteristics. However, it was not clear if PB was required for aggregation of viruses or if it could be replaced by a different cationic polymer. To address this point, we transduced cells with virus stocks containing 640 μg/mL of CSC and a range of concentrations of each of the cationic polymers listed in Table 4.1. We found that in each case transduction was enhanced (Figure 4.9).

These results indicated that varying the molecular weight, charge density, charged groups or geometry of the polymers did not prevent viruses from aggregating into complexes and efficiently transducing cells (for a more detailed analysis of the trends observed, refer to the discussion section). To further examine if varying the structure of the polymers affected transduction, we determined the weight ratio of oppositely charged polymers that resulted in maximal gene transfer (Table 4.2). While maintaining this ratio, we varied the concentrations of the polymers and assessed their effect on transduction. In Table 4.2 we report the concentrations that achieved maximal transduction, as well as the fold enhancement in gene transfer with respect to transduction with virus stocks containing 8 μg/mL of PB. The fold-increase in gene transfer varied between 2.5 and 5.5 depending on the combination of polymers used. We also estimated the ratio of positive to negative charges that were added to the medium in order to achieve maximal transduction. This ratio was close to 1:1 and varied between 0.78 and 1.53 depending on the combination of polymers used (Table 4.2). In most cases, the positive charge was in slight excess of the negative charge.

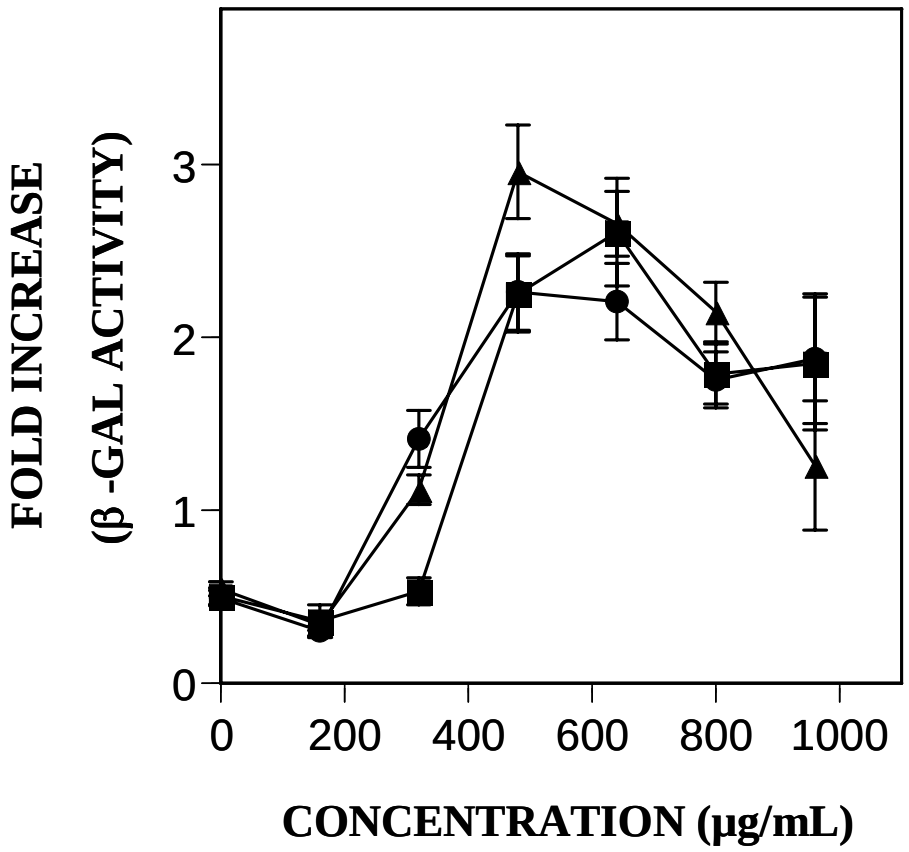

Figure 4.7 Combinations of PB and different anionic polymers enhance transduction. Virus stocks were brought to 640 μg/mL of PB and to various concentrations of CSA (■), CSB (●) or CSC (▲), then used to transduce NIH 3T3 cells. Each point represents the mean ± the standard deviation of three samples.

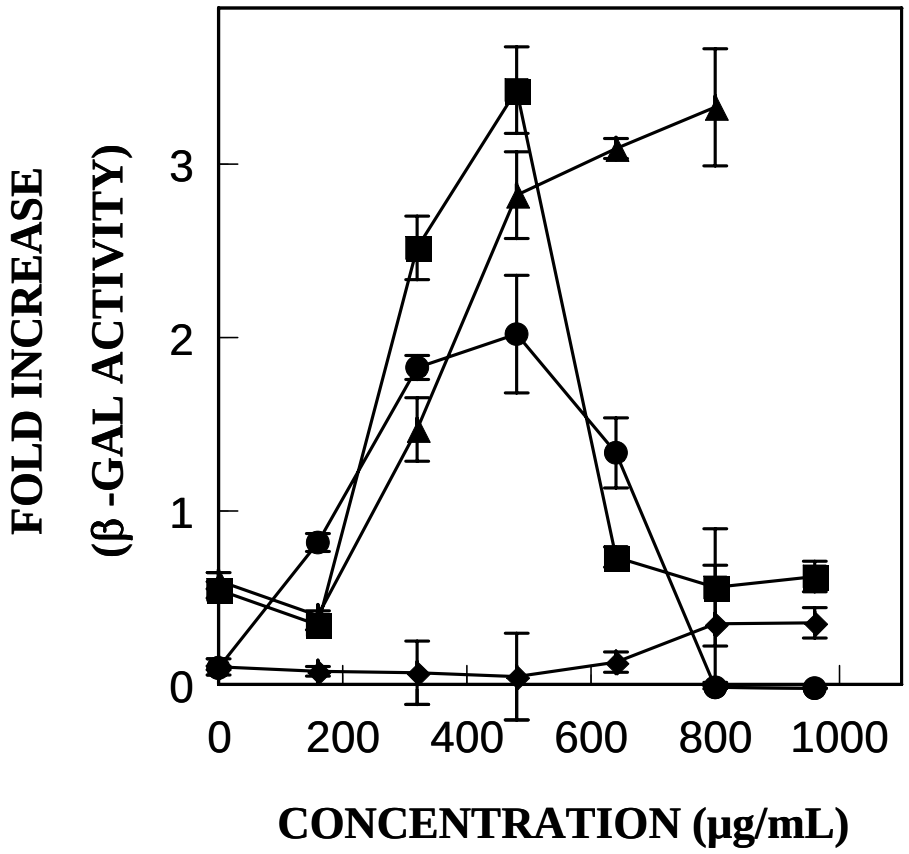

Figure 4.8 Combinations of PB and different anionic polymers enhance transduction (continue). Virus stocks were brought to 640 µg/mL of PB and to various concentrations of DS (■), HEP (●), desHEP (▲) or HA (◆), then used to transduce NIH 3T3 cells. Each point represents the mean ± the standard deviation of three samples.

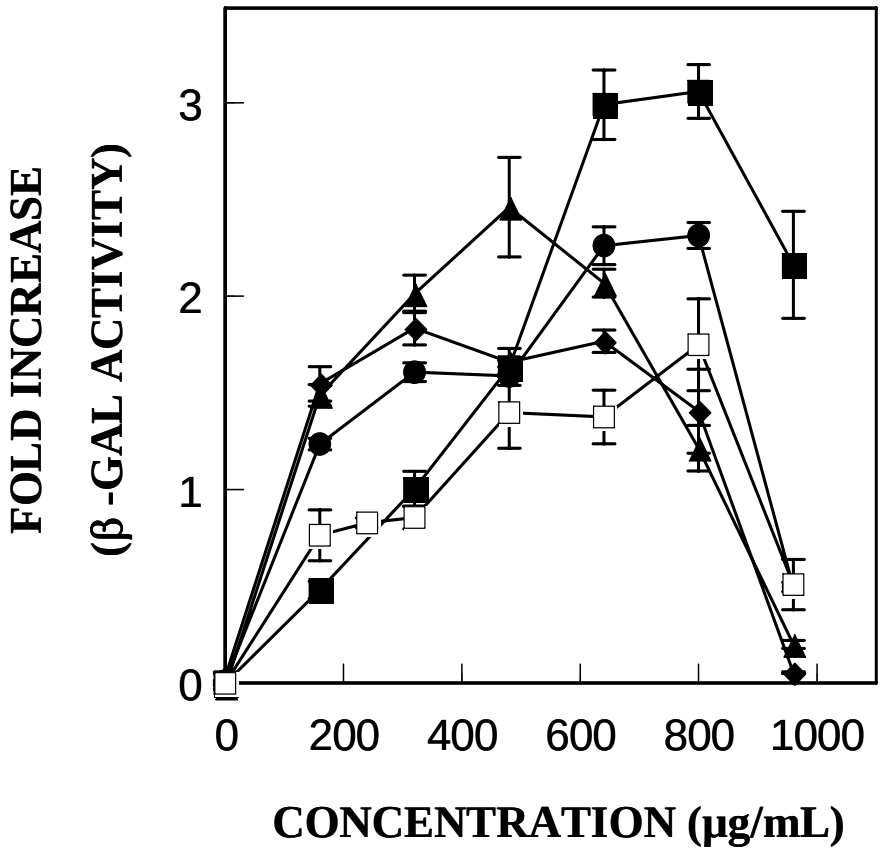

Figure 4.9 Combinations of CSC and different cationic polymers enhance transduction. Virus stocks were brought to 640 µg/mL of CSC and to various concentrations of PB (■), PLL (low) (●), PLL (med) (▲), PLL (high) (◆) or PS (□), then used to transduce NIH 3T3 cells. Each point represents the mean ± the standard deviation of three samples.

Table 4.2 Combinations of oppositely charged polymers that maximize gene transfer

Cationic Polymer	Anionic Polymer	Optimum ratio cationic : anionic[a]	Charge ratio positive : negative[b]	Optimum concentration (μg/mL)[b]	Fold increase relative to 8 μg/mL of PB[c]
PB	DS	4 : 3	1 : 1.24	160:120 - 320:240	5.48 ± 1.58
PB	HEP	4 : 3	1 : 0.87	160:120 - 640:480	4.10 ± 1.03
PB	desHEP	2 : 3	1 : 1.53	160:240 - 640:960	4.18 ± 0.24
PB	HA	1 : 1	1 : 0.45	80:80 - 160:160	2.99 ± 0.41
PB	CSA	1 : 1	1 : 0.78	320:320 - 640:640	3.49 ± 0.78
PB	CSB	1 : 1	1 : 0.78	320:320	3.52 ± 0.48
PB	CSC	1 : 1	1 : 0.78	320:320	4.00 ± 0.13
PLL (low)	CSC	1 : 1	1: 0.87	200:200 - 320:320	4.88 ± 0.88
PLL (med)	CSC	1 : 1	1: 0.87	160:160	3.94 ± 0.74
PLL (high)	CSC	1 : 1	1: 0.87	40:40 -160:160	2.71 ± 0.49
PS	CSC	1 : 1	N/A	320:320 - 640:640	2.45 ± 0.65

a: Optimum ratio based on weight concentration
b: Corresponding to the optimum weight ratio of cationic polymer to anionic polymer. Estimated based on the number of charged groups and molecular weight of the monomers in each polymer.
c: Results obtained from three independent experiments
N/A: not available. Only the composition of a major component of PS was available.

Based on our finding that viruses interacted with the cationic polymer and not with the anionic polymer, we considered the possibility that this slight excess of positive charge was neutralized by negative charges on the surface of virus particles. To further explore this possibility, we hypothesized that an excess of negative charge in the medium would prevent viruses from incorporating into complexes. To test this hypothesis, we brought virus stocks to 80 μg/mL of PB and to a range of concentrations of CSC, and then incubated the stocks for 20 min. We centrifuged each stock and obtained visible pellets when 80 to 960 μg/mL of CSC were used, suggesting that polyelectrolyte complexes were formed even when CSC was in large excess. To determine the extent to which viruses aggregated within these complexes, we measured the concentration of p30 in the original stocks, the supernatants and the pellets. About 70% of p30 was recovered in the pellet when the concentration of CSC was 80 μg/mL, that is, when the two polymers were added at the optimum ratio 1:1. However, when concentrations of CSC equal to or higher than 160 μg/mL were used, most of the viral proteins remained in the supernatant (Figure 4.10), indicating that viruses did not aggregate with polyelectrolyte complexes. This result suggested that viruses competed with the anionic polymer for binding to the cationic polymer, and that an excess of cationic polymer was required to secure incorporation of viruses into polyelectrolyte complexes.

In previous studies we observed that viruses were located primarily on the outer surfaces of the complexes (refer to Chapter 3)[15]. This observation raised the possibility that the oppositely charged polymers were forming cores and that viruses were binding to the surface of these cores. However, the results from single flocculation indicated that viruses could bind to free cationic polymer and thus raised the possibility that flocculation occurred through a patching and bridging mechanism [31]. According to this mechanism,

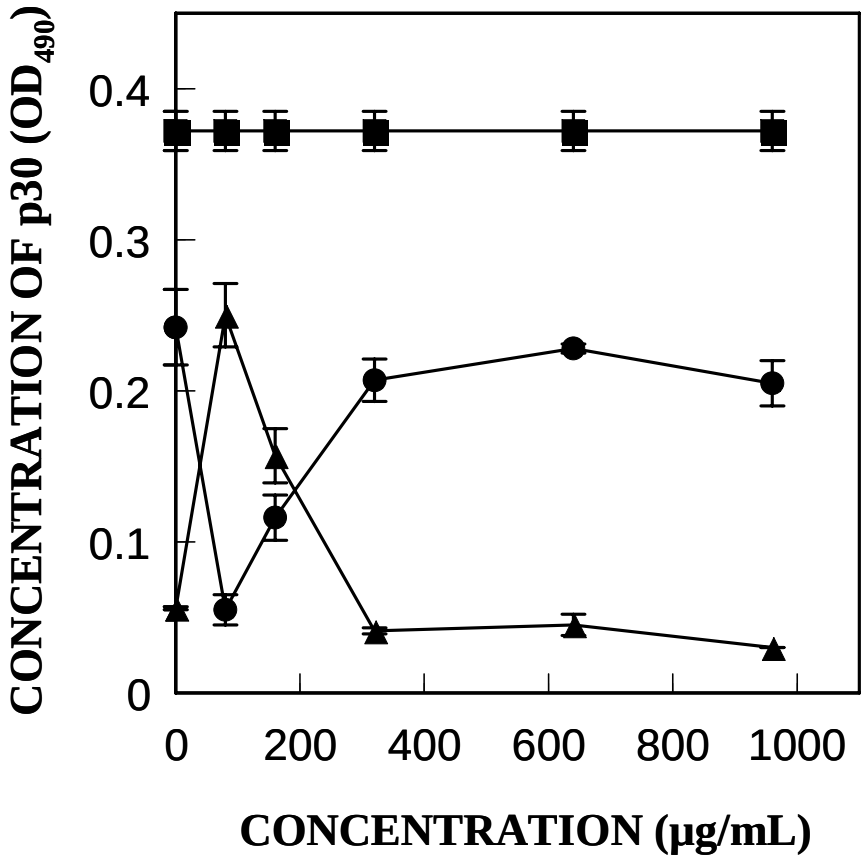

Figure 4.10 An excess of negative charge prevents incorporation of viruses into complexes. Virus stocks were brought to 80 μg/mL of PB and to various concentrations of CSC, incubated for 20 min at 37°C, then centrifuged for 5 min at 10000g. The supernatants were decanted and the pellets resuspended to the original volume in fresh medium. The concentration of p30 in the original solutions (■), the supernatants (●), and the resuspended pellets (▲) was quantified by ELISA. Each point represents the mean ± the standard deviation of three samples.

the cationic polymer would bind to the negatively charged surface of the viruses and render this surface positively charged. Then, the anionic polymer would form bridges among virus particles. It has been shown that the order of addition of polymers determines the efficiency of patching and bridging [32, 33]. In our system, a more efficient patching and bridging was expected if the cationic polymer was added prior to the anionic polymer. To determine if this was the case, we brought a virus stock to 80 μg/mL of PB, incubated it for 10 min at 37°C, then brought it to 80 μg/mL of CSC and incubated it for another 10 min at 37°C. As a control, we brought the stock to 80 μg/mL of CSC, incubated it for 10 min at 37°C, then brought it to 80 μg/mL of PB and incubated it for another 10 min at 37°C. As an additional control, we brought the stock to 80 μg/mL of PB and to 80 μg/mL CSC by adding the polymers in immediate succession, and then incubated it for 20 min at 37°C. We centrifuged the stocks for 5 min at 10000g, decanted the supernatants and resuspended the pellets to their original volume in fresh medium. We found that 70 to 80% of the virus capsid protein (p30) was recovered in the pellet regardless of the order of addition of the polymers to the virus stock (Figure 4.11). Moreover, the recovery of p30 was slightly lower when PB was added before CSC than when CSC was added before PB or when the polymers were added in immediate succession. These results suggested that flocculation of viruses did not occur through a patching and bridging mechanism. Probably, PB and CSC were aggregating into complexes and viruses were binding to preformed complexes of PB and CSC (refer to discussion for more detail).

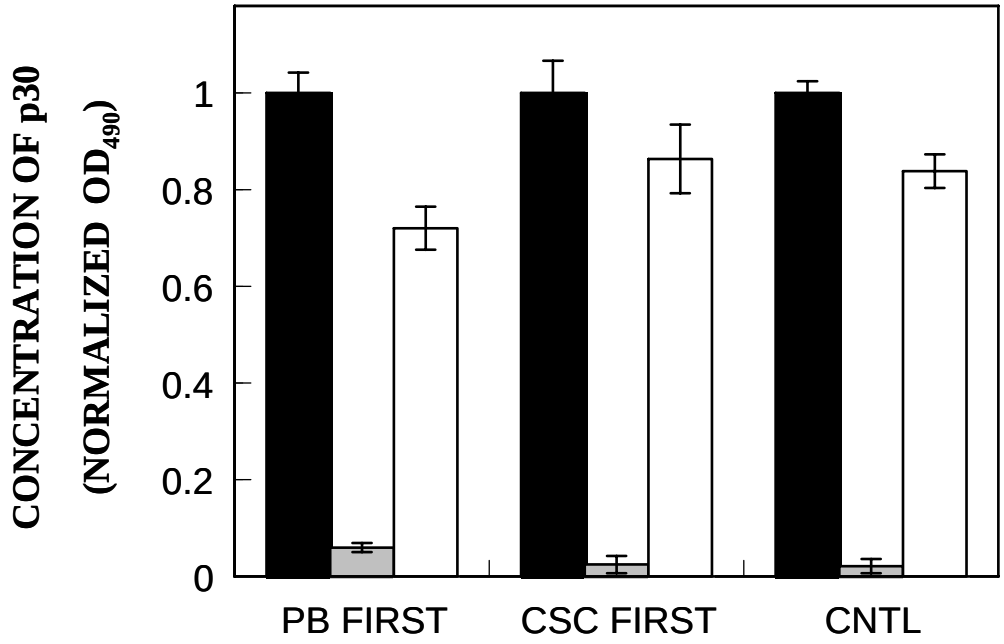

Figure 4.11 The order of addition of the polymers does not affect the efficiency of flocculation. Virus stocks were brought to 80 μg/mL of PB, incubated at 37 °C for 10 min, then brought to 80 μg/mL of CSC and incubated at 37 °C for 10 min (PB FIRST). Alternatively, virus stocks were brought to 80 μg/mL of CSC, incubated at 37 °C for 10 min, then brought to 80 μg/mL of PB and incubated at 37 °C for 10 min (CSC FIRST). As a control, virus stocks were brought to 80 μg/m of PB and to 80 μg/mL of CSC by addition of the polymers in immediate succession, then incubated at 37 °C for 20 min (CNTL). The stocks were centrifuged for 5 min at 10000g, the supernatants decanted and the pellets resuspended to the original volume in fresh medium. The concentration of p30 in the original stocks (black bars), the supernatants (gray bars) and the pellets (white bars) was measured by ELISA. Each point represents the mean ± the standard deviation of three samples.

Single flocculation vs. dual flocculation for enhancing gene transfer

We have previously used dual flocculation of viruses with the purpose of enhancing gene transfer by rapidly transporting viruses to the surface of the cells (refer to Chapter 3) [15] and by concentrating virus stocks (refer to Chapter 2) [14].

We have shown that viruses within complexes of oppositely charged polymers are rapidly transported to the surface of the cells, and that this rapid transport correlates with increases in the efficiency of transduction. Our results from Figure 4.1 indicated that single flocculation of viruses with PB also increased the rate of binding of viruses to cells, and therefore raised the possibility that single flocculation could enhance gene transfer. However, we have also observed that high doses of PB are cytotoxic (Figure 4.3), and therefore not suitable for transduction. Given that in previous experiments we assessed the toxicity of PB after a two-day transduction, we considered the possibility that PB could be used for short-term transductions. To address this possibility, we incubated cells for various periods of time with virus stocks containing 320 μg/mL of PB. As controls, we also incubated cells with virus stocks containing 320 μg/mL of CSC, a combination of 320 μg/mL of PB and 320 μg/mL of CSC, or 8 μg/mL of PB. We found that exposure to CSC, to a combination of PB and CSC or to 8 μg/mL of PB did not decrease cell viability. In contrast, exposure to high concentrations of PB reduced cell viability by 10% after 8 hours, by 66% after 14 hours and to undetectable levels after 30 hours (Figure 4.12). In parallel, we quantified the efficiency of gene transfer by measuring the β-galactosidase activity inside the cells. In the presence of only CSC, transduction was below the sensitivity of the assay. In the presence of 320 μg/mL of PB, transduction increased at early times, but remained lower than transduction in the presence of both PB and CSC, and decreased to undetectable levels after 10 hrs of incubation with cells. When both polymers were used, the efficiency of transduction was

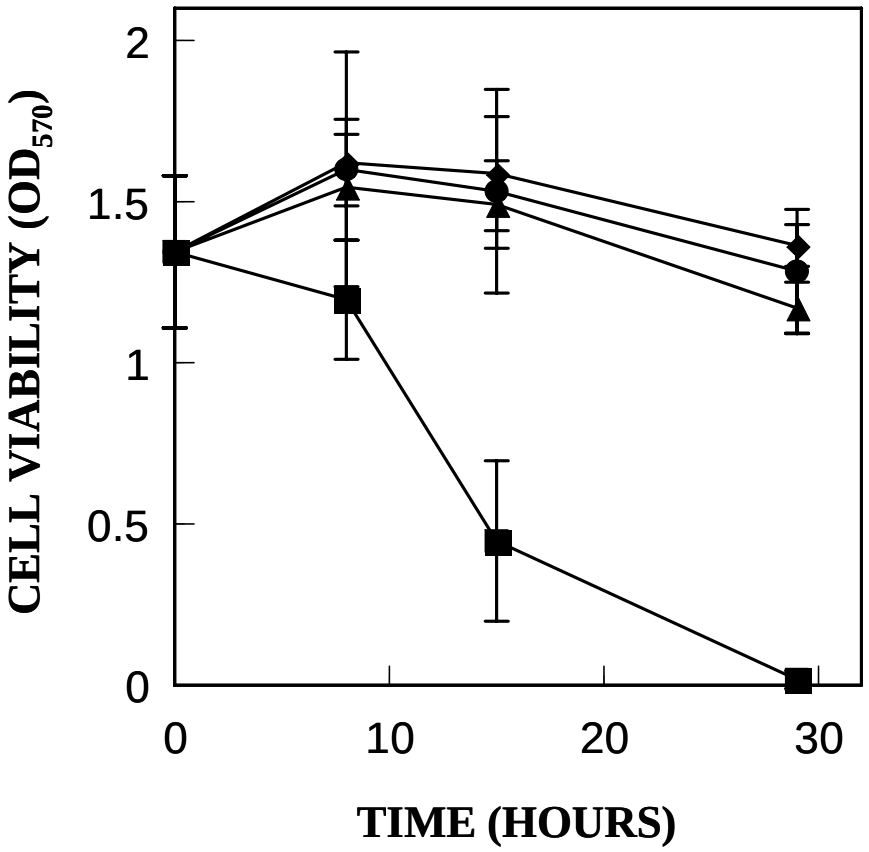

Figure 4.12 High concentrations of PB, but not CSC, are toxic to target cells. Virus stocks were brought to 320 μg/mL of PB (■), 320 μg/mL of CSC (●), to a combination of 320 μg/mL of PB and 320 μg/mL of CSC (▲), or to 8 μg/mL of PB (◆), then added to NIH 3T3 fibroblasts. At various times, the conditioned medium was replaced with fresh medium. Two days later, cell viability was quantified. Each point represents the mean ± the standard deviation of three samples.

higher than that in the presence of 8 μg/mL of PB (Figure 4.13). These results indicated that single flocculation could only be used in transductions for less than 8 hours and that, even within this time frame, transduction with two oppositely charged polymers was more efficient.

We have also used dual flocculation to concentrate virus stocks (refer to Chapter 2) [14]. Briefly, we induce flocculation of viruses in the presence of oppositely charged polymers, centrifuge the stock and concentrate the complexes into a pellet. We decant the supernatant, resuspend the pellet to a smaller volume and use it to transduce cells. Our findings from Figures 4.2 and 4.3 indicated that flocculation with a single species of cationic polymer permited concentrating virus aggregates into a pellet and that the pellet was not cytotoxic. This suggested that single flocculation of viruses could be used for concentration of virus stocks prior to transduction. To evaluate this possibility, we brought virus stocks to 320 μg/mL of PB, or as a control to a combination of 80 μg/mL of PB and 80 μg/mL of CSC. We pelleted the stocks and resuspended the pellets in fresh medium to the original volume or to one fourth the original volume. We transduced cells with these resuspended pellets or, as a control, with virus stocks containing 8 μg/mL of PB. Resuspension of the pellet to the original volume did not enhance gene transfer with respect to the control when single flocculation was used, but enhanced gene transfer by 4.5-fold when double flocculation was used (Figure 4.14). Upon a four-fold concentration of the stock, single flocculation led to a 2.4-fold increase in transduction, while dual flocculation led to a 13-fold increase in transduction.

These differences in transduction were likely due to differences in the rate that viruses bound to cells. Viruses concentrated after single flocculation were probably transported to the cells in a similar manner as free viruses, while viruses concentrated after double flocculation reached the cells much faster than by simple diffusion. To

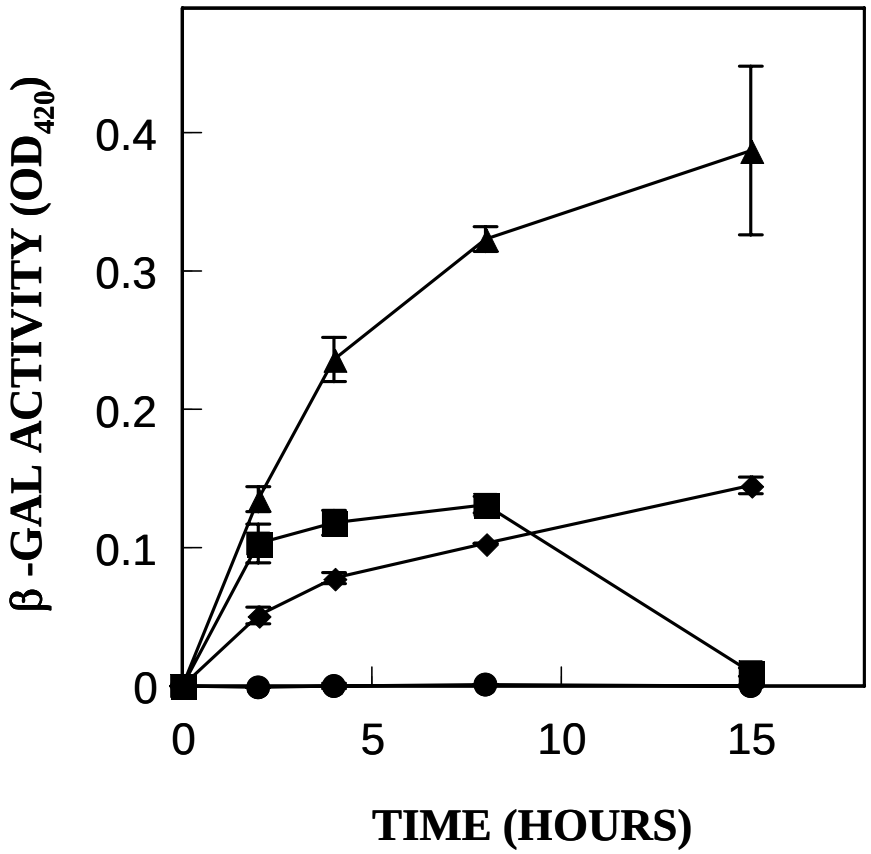

Figure 4.13 High concentrations of PB enhance gene transfer in short-term transductions. Virus stocks were brought to 320 μg/mL of PB (■), 320 μg/mL of CSC (●), to a combination of 320 μg/mL of PB and 320 μg/mL of CSC (▲), or to 8 μg/mL of PB (◆), then added to NIH 3T3 fibroblasts. At various times, the conditioned medium was replaced with fresh medium. Two days later, the efficiency of transduction was quantified. Each point represents the mean ± the standard deviation of three samples.

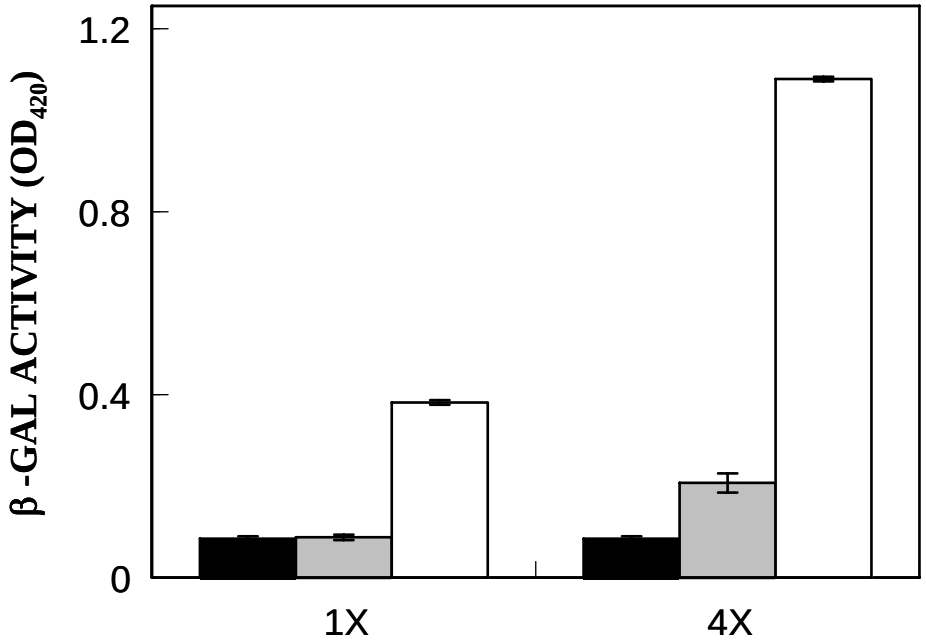

Figure 4.14 Viruses concentrated after dual flocculation achieve higher efficiencies of transduction than viruses concentrated after single flocculation. Virus stocks were brought to 320 μg/mL of PB (gray bars) or to a combination of 80 μg/mL of PB and 80 μg/mL of CSC (white bars), incubated at 37°C for 20 min, then centrifuged for 5 min at 10000g. The supernatants were decanted and the pellets resuspended in fresh medium to the original volume (1X) or to one fourth the original volume (4X). NIH 3T3 cells were transduced with the resuspended pellets, or a control, with virus stocks containing only 8 μg/mL of PB (black bars). Each point represents the mean ± the standard deviation of three samples.

assess this possibility, we measured the kinetics of virus binding. We added the pellets that had been resuspended to the original volume to layers of cells. After various intervals, we collected the supernatant and replaced it with fresh medium. We measured the concentration of p30 remaining in the supernatant over time, as well as the kinetics of gene transfer. The rates of binding and transduction with viruses concentrated after single flocculation were similar to those from the original virus stock containing 8 μg/mL of PB (Figures 4.15 - 4.16). On the contrary, the rates of binding and transduction with viruses concentrated after double flocculation were higher than those from the original virus stock containing 8 μg/mL of PB.

4.5 Discussion

We have previously shown that the combined addition of the cationic polymer PB and the anionic polymer CSC to stocks of retroviruses results in flocculation of virus particles and in substantial enhancement of transduction. In this study we showed that it is possible to induce flocculation of viruses and enhance gene transfer using other combinations of oppositely charged polymers. During dual flocculation, viruses appear to bind primarily to the cationic polymer, as flocculation with a single species of cationic polymer, but not with single species of anionic polymer, was possible. The anionic polymer appears to also bind to the cationic polymer, mitigate the cytotoxicity due to the excess of positive charge in the medium, and give rise to complexes bigger than those obtained upon single flocculation.

Based on these findings, we examined the possibility that the cationic polymer could form patches on the surface of virus particles, while the anionic polymer could form bridges to connect the positively charged patches. This mechanism of patching and bridging has been proposed for dual flocculation of charged particles, namely fine clay

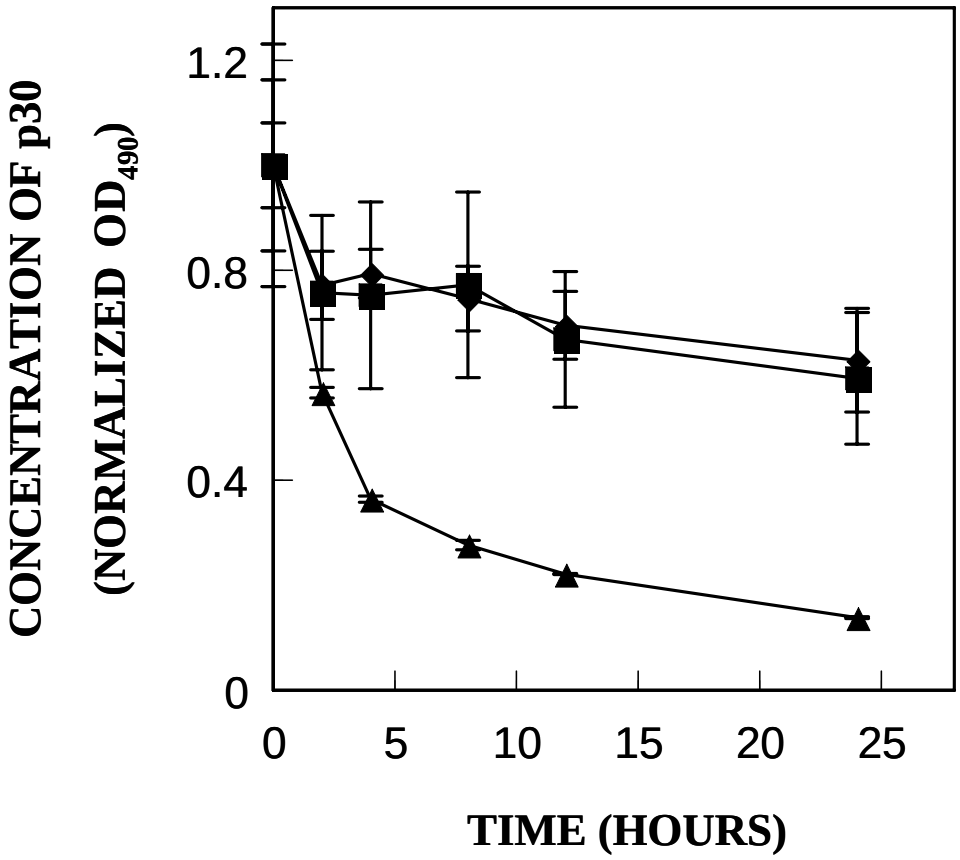

Figure 4.15 Viruses concentrated after single flocculation with PB bind to the cells at the same rate as viruses in the original stock. Virus stocks were brought to 320 μg/mL of PB (single flocculation) or to a combination of 80 μg/mL of PB and 80 μg/mL of CSC (dual flocculation), incubated at 37°C for 20 min, then centrifuged for 5 min at 10000g. The supernatants were decanted and the pellets resuspended to the original volume in fresh medium. The resuspended pellet obtained after single flocculation (■), the resuspended pellet obtained after dual flocculation (▲) or the original stock containing 8 μg/mL of PB (◆) were added to NIH 3T3 cells. At various times, the supernatant was collected and the concentration of virus capsid protein (p30) remaining in the supernatant was quantified by ELISA. Each point represents the mean ± the standard deviation of three samples.

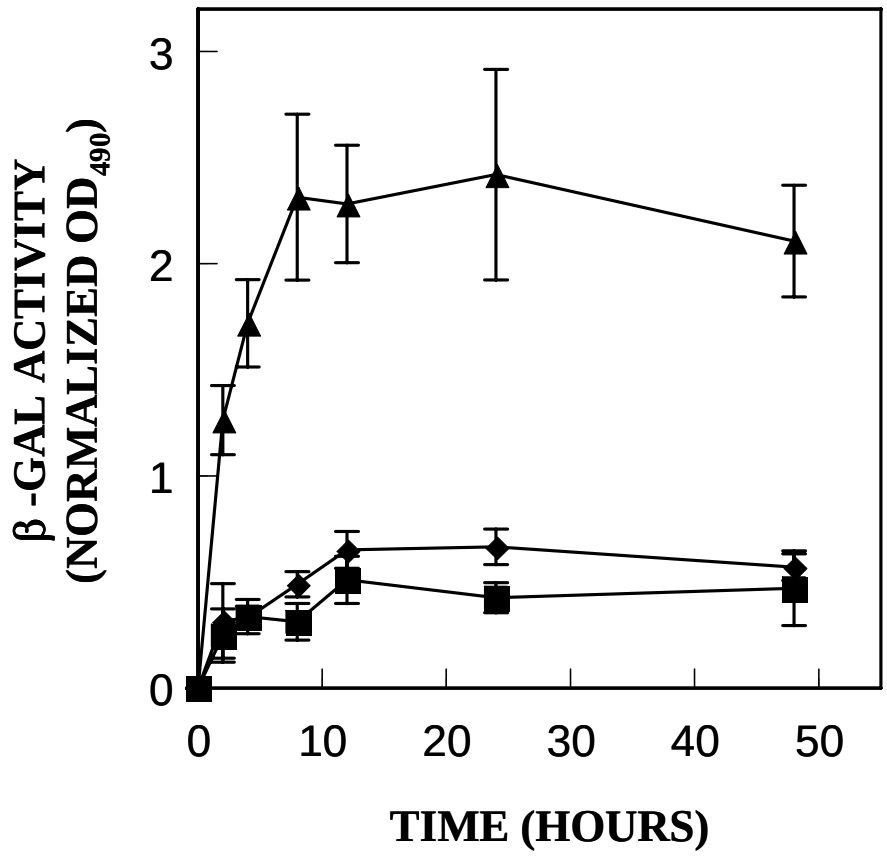

Figure 4.16 Viruses concentrated after single flocculation with PB transduce at the same rate as viruses in the original stock. Virus stocks were brought to 320 μg/mL of PB (single flocculation) or to a combination of 80 μg/mL of PB and 80 μg/mL of CSC (dual flocculation), incubated at 37°C for 20 min, then centrifuged for 5 min at 10000g. The supernatants were decanted and the pellets resuspended to the original volume in fresh medium. The resuspended pellet obtained after single flocculation (■), the resuspended pellet obtained after dual flocculation (▲) or the original stock containing 8 μg/mL of PB (◆) were used to transduce NIH 3T3 cells. At various times after the start of transduction, the conditioned medium was replaced with fresh medium. Two days later, the efficiency of transduction was quantified. Transduction was normalized with the concentration of p30 in each virus stock. Each point represents the mean ± the standard deviation of three samples.

particles and alumina particles [32-34] and for flocculation of Escherichia coli and B. thuringiensis var. israelensis cellular suspensions [35]. However, flocculation by this mechanism is more efficient when the polymer that forms the patches is mixed with the colloidal particles prior to addition of the oppositely charged polymer [32, 33]. In our study, addition of the cationic polymer prior to addition of the anionic polymer did not improve the efficiency of virus aggregation with respect to simultaneous addition of both polymers. Moreover, patching and bridging would most likely result in incorporation of viruses in the interior of the complexes, which is not consistent with our previous observations. Upon double flocculation, viruses within complexes of PB and CSC are located primarily on their outer surfaces of the complexes (refer to Chapter 3)[15].

Based on these findings, we propose that flocculation of viruses with oppositely charged polymers occurs as a two step process. First, oppositely charged polymers aggregate into polyelectrolyte cores, then the viruses bind to the surface of these cores. In this model, the oppositely charged polymers aggregate through electrostatic interactions, thereby neutralizing most of their charges and giving rise to hydrophobic structures. Molecules of the positively charged polymer, which is added in excess, are exposed at the surface of these structures and act as anchor for virus particles (Figure 4.17).

The first step of this model is consistent with a model proposed by Schatz et al. to characterize the aggregation of the polyanion dextran sulfate with the polycation chitosan [36, 37]. They showed that addition of dextran sulfate to a solution of chitosan results in the formation of spherical particles, which are likely composed by a neutral core surrounded by a shell of uncomplexed chitosan. The second step of this model can be described as aggregation of viruses into preformed complexes. Preformed complexes of oppositely charged polymers have proven efficient at flocculating clay and silica particles, if composed of non-stoichiometric ratios of the anionic and the cationic polymer

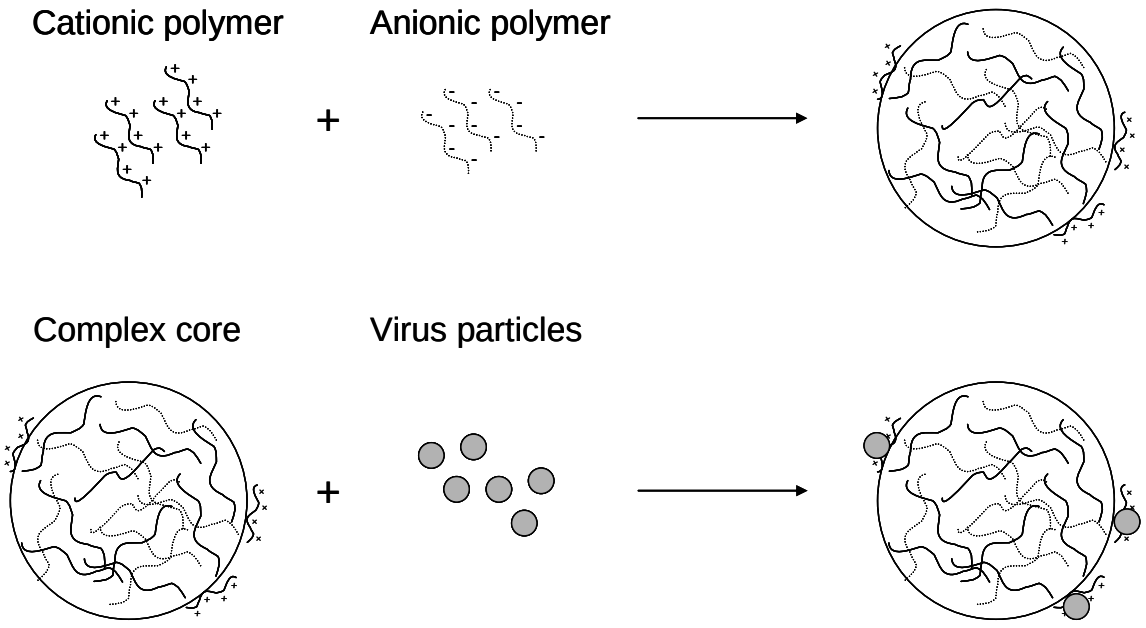

Figure 4.17 Model for dual flocculation of viruses with oppositely charged polymers. The anionic and the cationic polymer aggregate and form a hydrophobic core. The excess of cationic polymer bind to the surface of this core and form positively charged patches. Viruses bind to these patches.

[32, 38-40]. In our system, we did not preform polyelectrolyte complexes, but mixed the oppositely charged polymers and the viruses in a single step. It is possible that the cationic polymer is more readily attracted to the anionic polymer than to virus particles, such that aggregation of viruses can only occur once polyelectrolyte complexes are formed. Alternatively, the anionic polymer might be in vast excess to the virus particles, such that most of the cationic polymer will bind to the anionic polymer and only the leftover cationic polymer will bind to the viruses.

Also, it is to be expected that viruses, due to their charged surface, would preferentially bind to the hydrophilic outer surface of the complexes and not to the hydrophobic core. Consistent with this idea, Cathrin et al. have shown that while hybrophobic bacteria are found in the interior of sludge flocs, hydrophilic bacteria are found at the surface of these flocs [41].

Previous studies of dual flocculation have shown that varying the characteristics of the polymers can have a major impact on the efficiency of flocculation of colloidal particles [31, 32, 38-40]. In this study, we did not directly evaluate the efficiency of flocculation by measuring the concentration of virus particles within the polyelectrolyte complexes. Rather, we evaluated the extent to which flocculation with charged polymers enhanced gene transfer by rapidly transporting viruses to the surface of the cells. Regardless of the combination of cationic polymer and anionic polymer we added to the virus stocks, gene transfer was significantly enhanced and the range of enhancement varied between 2.5 and 5.5-fold with respect to a standard transduction in the presence of 8 μg/mL of PB. We concluded that varying the nature of the charged groups, the charge density, the molecular weight or the geometry of the polymers, did not prevent viruses from incorporating into complexes. However, enhancement of gene transfer appeared to benefit from the use of lower molecular weight cationic polymers. In our study, the efficiency of transduction increased with decreasing molecular weight of PLL

in complexes of PLL and CSC. Interestingly, increasing the molecular weight of PLL also decreased the ability of CSC to mitigate the cytotoxicity in the medium. Additionally, we noted that the high molecular weight anionic polymer HA was less efficient than other, lower molecular weight, anionic polymers at mitigating the toxicity of PB and at enhancing gene transfer. These findings suggest that low molecular weight polymers are more likely to aggregate into polyelectrolyte complexes and favor flocculation of viruses. Nevertheless, it is possible that factors other than molecular weight affected flocculation. For example, HA was the only non-sulfated anionic polymer we tested and it is possible that sulfate groups favor aggregation of viruses. In order to identify the precise factors that assist flocculation, we need to conduct a more direct and exhaustive analysis of the physicochemical characteristics of the complexes.

Regardless of the specific characteristics of the polymers we used, in almost all the cases we achieved maximal gene transfer when the positive charges added to the medium were in slight excess of the negative charges. This finding is consistent with our flocculation model (Figure 4.17). However, it is not clear why an excess of negative charge was necessary for two of the polymer combinations, namely PB with DS and PB with desHEP. It is possible that DS, due to its branched conformation and its high molecular weight, imposed electrosteric hindrances on the complexes and did not efficiently aggregate with other polymer chains into compact structures. In the case of desHEP, it is possible that a high degree of acetylation resulted in higher hydrophobicity of the polymer, which impeded its efficient complexation with CSC by charge-charge interactions.

While conducting this study, we found that single flocculation of viruses with concentrations of PB as high as 320 μg/mL induced aggregation of more than 70% of the viruses. However, only a small fraction of PB interacted with virus particles, while the rest remained free in solution and was highly cytotoxic. We were able to purify the virus

aggregates from free PB by pelleting the complexes, removing the supernatant and resuspending the pellet in fresh medium. Interestingly, after resuspension, viruses were transported and transduced at similar rates as free virus particles, suggesting that they had disaggregated from the complexes. These viruses were still bioactive and enhanced gene transfer when resuspended to one fourth the original volume. These findings indicate that single flocculation is an efficient method for concentrating virus stocks and improving transduction. Nevertheless, concentration after dual flocculation achieved levels of gene transfer 4 to 5-fold higher than concentration after single flocculation. This higher increase in transduction was likely due to the fact that viruses within complexes of PB and CSC do not disaggregate upon resuspension of the pellet in fresh medium. They reach the surface of target cells much faster than free viruses, thereby increasing the rate of transduction (refer to Chapter 3) [15].

Taken together, our results suggest that concentration of viruses after double flocculation has the advantage of increasing the rate of binding of viruses to cells. On the other hand, concentration of viruses after single flocculation has the advantage of concentrating only trace doses of polymer, and is therefore desirable for applications where an excess of polymers is undesirable or for which higher rates of virus adsorption due to sedimentation are not necessary.

4.6 References

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CHAPTER 5

COMPLEXATION WITH CHONDROITIN SULFATE C AND POLYBRENE RAPIDLY PURIFIES RETROVIRUS FROM INHIBITORS OF TRANSDUCTION AND SUBSTANTIALLY ENHANCES GENE TRANSFER

5.1 Abstract

Using amphotropic retrovirus stocks produced by TELCeB6-A cells that encode the Escherichia coli *lacZ* gene, we found that complexation with chondroitin sulfate C (CSC) and Polybrene (PB) is an effective means to purify retrovirus. Virus stocks contained high levels of inhibitory activity that blocked amphotropic, but not ecotropic, retrovirus transduction. When virus stocks were brought to 80 μg/mL each of CSC and PB, complexes of CSC and PB formed that incorporated more than 70% of the virus particles but less than 0.4% of all other proteins and no detectable inhibitory activity. Purified virus transduced NIH 3T3 murine fibroblasts 21 to 186-fold more efficiently than virus that was not purified. In addition, virus purification significantly altered the dose response of transduction. When virus that had not been purified was used to transduce cells, the relationship between transduction and virus concentration was highly non-linear. In contrast, when purified virus was used, transduction increased monotonically and was linearly proportional to virus concentration, except when high doses of virus were used. Interestingly, when high doses of virus were used gene transfer reached a maximum plateau level, most likely because particle-associated amphotropic envelope proteins had saturated the cellular receptors for the virus. Our findings illustrate that retrovirus purification increases the maximum number of genes that can be transferred, reduces the amount of virus required to achieve a given level of gene transfer, and

reduces uncertainties about the relationship between the amount of virus used and the number of genes transferred.

5.2 Introduction

Recombinant retroviruses are frequently used to genetically modify cells, primarily because they permanently and faithfully integrate their genetic material into the chromosomal DNA of target cells [1]. Unfortunately, the use of retroviruses in the clinic has met with limited success, in part because retroviruses often transfer too few genes to cells to achieve the desired therapeutic effect [2]. In addition, it has proven difficult to achieve predictable and reproducible levels of gene transfer with retroviruses [3]. Predictable and reproducible levels of gene transfer are important in order to maximize the benefits of retrovirus transduction while minimizing the likelihood of any adverse side effects, including insertional mutagenesis [3, 4]. We and others have found that one cause of low and variable levels of gene transfer is the presence of inhibitors of transduction in virus stocks [5-9]. A number of substances that inhibit transduction have been identified, including negatively charged molecules such as proteoglycans and glycosaminoglycans [5, 6], as well as retrovirus envelope proteins that are not associated with the virus particles (i.e., free envelope proteins) [5, 6, 9, 10]. Little is known about the mechanism of inhibition, or what controls whether or not inhibitors are present in virus stocks, or to what extent these inhibitors block transduction when they are present. As a result, the presence of inhibitory activity in virus stocks makes it difficult to predict and control the outcome of retrovirus transduction protocols.

Traditionally, predictions of the outcome of transduction protocols have been based on the results of virus titer assays. Virus titer assays provide an estimate of the concentration of active virus in virus stocks. To determine titer, virus stocks are diluted several thousand-fold and then used to transduce cells [11-13]. The titer, expressed as

the number of colony forming units per milliliter (CFU/mL), is the number of colonies of transduced cells multiplied by the dilution factor and divided by the volume of virus that was applied to the cells. Values for titer are frequently incorporated into the calculation of the multiplicity of infection (MOI), the expected number of gene transfer events per cell in a transduction protocol. Unfortunately, recent studies have demonstrated that virus titer and MOI are not reliable predictors of the transduction process [14, 15]. This is due in part to the fact that virus titers are measured using virus stocks that have been diluted several thousand-fold in fresh medium and therefore contain virtually no inhibitory activity. Under these conditions the number of genes transferred is proportional to the concentration of active virus. In contrast, when high doses of virus are applied to cells, as is done in human gene transfer protocols, the relationship between the concentration of active virus and the number of genes transferred is non-linear and complex, due in part to the presence of high levels of inhibitory activity [9, 14].

As a result, there is significant motivation for developing methods for purifying retroviruses from inhibitors of transduction. One of the most commonly used methods for purifying retroviruses is to pellet them by ultracentrifugation, after which they are resuspended in fresh cell culture medium that does not contain any inhibitors. Unfortunately, retroviruses are often inactivated by this procedure, presumably because shear forces cause the retrovirus envelope proteins to separate from the virus particles, giving rise to virus particles that are no longer fusogenic. The problem of inactivation of retroviruses by ultracentrifugation was solved to some extent with the development of retroviruses pseudotyped with the vesicular stomatitis virus G protein (VSV-G), which are not inactivated by ultracentrifugation [16]. Unfortunately, the cytotoxicity of the VSV-G protein has complicated attempts to construct stable virus producer cell lines and limits the amount of virus that can be applied to the target cells [17-19]. VSV-G pseudotyped retroviruses have other shortcomings as well, including the fact that they

are pantropic [16] and are therefore less suitable than most other retrovirus pseudotypes for use in applications that require targeted gene delivery. In addition, despite their exceptionally wide tropism, VSV-G pseudotyped retroviruses are not able to efficiently transduce some important cell types, including polarized epithelial cells of the lung [20].

Virus purification strategies are needed that will work with all types of retrovirus pseudotypes, not just with retroviruses that can be concentrated by ultracentrifugation. Numerous alternatives to ultracentrifugation as a means to purify retrovirus have been developed, including low-speed centrifugation, size-exclusion membrane filtration, affinity chromatography, and complexation with calcium phosphate, cationic polymers or paramagnetic beads [11, 12, 16, 21-33]. Although each of these approaches are capable of concentrating retrovirus stocks and improving gene transfer, they do not increase the number of genes transferred to the same extent that the virus stocks are concentrated, which suggests that these virus processing methods co-concentrate inhibitors [7, 34] or directly reduce the ability of the viruses to transduce cells. In addition, many of these methods are labor and time intensive and difficult to scale up.

Improved methods for processing retrovirus stocks are needed that are rapid, inexpensive, scalable, universally applicable to any type of retrovirus, and which do not co-concentrate inhibitors or otherwise reduce the ability of the viruses to transduce cells. Recently, we developed a rapid method to concentrate retrovirus stocks [13, 35] that consists of adding high concentrations of the oppositely charged polymers Polybrene (PB) and chondroitin sulfate C (CSC) to retrovirus stocks prior to transduction. Polymers and viruses flocculate and form high molecular weight complexes which can be rapidly pelleted by centrifugation in a table top centrifuge. Resuspension of the pellet to smaller volumes substantially increases the efficiency of gene transfer to numerous cell lines, as well as to primary human fibroblasts [13, 35, 36] and murine hematopoietic progenitor cells (personal communication, Spencer, HT, Emory University, 2005). In addition, we

observed that retroviruses, but few other proteins, were selectively incorporated into the polymer complexes (refer to Chapter 2) [13]. Given the selectivity of retrovirus incorporation, we hypothesized that complexation of retroviruses with PB and CSC could be used to purify them from inhibitors of transduction, increase the maximum level of gene transfer, and linearize the dose response curve, thereby simplifying attempts to obtain reproducible and predictable levels of transduction. To test this hypothesis, we formed retrovirus-polymer complexes in virus stocks produced by TELCeB6-A packaging cells that contained high levels of inhibitory activity, isolated the virus-polymer complexes, and examined the effect of this separation process on the dose response of gene transfer.

5.3 Materials and methods

Chemicals and antibodies. Chondroitin sulfate C (CSC) (shark cartilage), gluteraldehyde, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene, PB), o-Nitrophenyl β-D-galactopyranose (ONPG), 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and Igepal CA-630 were from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide 30%, bovine serum albumin fraction V (BSA) and Polyoxyethylene 20-Sorbitan Monolaurate (Tween 20) were from Fisher Scientific (Fair Lawn, NJ). Non-fat dry milk (blotting grade) was from Bio-Rad Laboratories (Hercules, CA). o-Phenylenediamine Dihydrochloride (OPD) was from Pierce (Rockford, IL). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Denville Scientific, Inc. (Metuchen, NJ). Mouse anti-p30 and mouse anti-gp70 antibodies were purified from the supernatant of the CRL-1219 (ATCC, Rockville, MD) and the 83A25 [37] hybridoma cell lines respectively, following standard procedures [38]. The goat polyclonal anti-p30 antibody (78S221) and the goat polyclonal anti-gp70 (79S834) were from Quality

Biotech (Camden, NJ). The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories (South San Francisco, CA).

Cell culture. NIH 3T3 mouse fibroblasts and the amphotropic packaging cell line Ψ-CRIP producing the α-SGC-*LacZ* virus [39] were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% bovine calf serum (Hyclone Labs Inc.), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Hyclone Labs Inc.) (DMEM/BCS). TELCeB6 (TE671 cells expressing Mo-MLVgagpol and the retroviral vector MFGnlsLacZ [40]) and HeLa cells (human adenocarcinoma cells, from the ATCC), were cultured in DMEM, 10% fetal bovine serum (Hyclone Labs Inc.), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (DMEM/FBS). RIE cells (rat intestinal epithelial cells), a kind gift from Donald H. Rubin, were cultured in DMEM, 10% fetal bovine serum (Hyclone Labs Inc.), 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2mM of L-glutamine (DMEM/FBS/L-GLUT).

Generation of a stable amphotropic retrovirus packaging cell line. An amphotropic packaging cell line (TELCeB6-A) was generated by stable transfection of TELCeB6 cells. Five micrograms of the plasmid FB4070ASALF, an expression plasmid that encodes for the amphotropic envelope glycoprotein (a kind gift of Stephen Russell), was dissolved in 400 μL of 0.25M $CaCl_2$, mixed with 400 μL of 2X HEPES buffered saline (274 mM NaCl, 42 mM Hepes acid, 10 mM KCl, 1.4 mM Na_2HPO_4 and 12 mM dextrose), incubated at room temperature for 20 minutes, then added to a 50% confluent T75 flask of TELCeB6 cells. Twelve hours after transfection the cells were washed with PBS, and then the medium replaced with fresh DMEM/FBS. Two days later the cells were trypsinized, pelleted, and resuspended. Two hundred microliters of the resuspended cells were diluted in 10 mL of selective medium (DMEM, 10% FBS, 50

μg/mL of phleomycin, and 7 μg/mL of blasticidin), and plated in a T75 flask. Fourteen days later pooled clones of stably transfected cells were frozen for later use.

Retrovirus production. To generate retrovirus stocks, virus-producing cells were grown to confluence in T175 tissue culture flasks, and then incubated for 24 h with 35 mL of cell culture medium. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use.

Diluted titer assay. Ten-fold serial dilutions of *lacZ* virus stock were made in DMEM/BCS and Polybrene (8 μg/mL). A 1-mL amount per well was used to transduce 3T3 cells that had been seeded (7 x 10^4 per well) the previous day in a 12-well plate. Two days after the start of the transduction, the cells were fixed and stained for β-galactosidase activity with X-Gal [41]. Colonies of *lacZ*$^+$ cells (typically in clusters of 2, 4, or 8 blue cells) were counted with the aid of a dissecting microscope. At appropriate dilutions of the virus stock, the clusters of blue cells were sufficiently spread over the dish such that each cluster arose from a single transduction event. From triplicate wells, the number of *lacZ*$^+$ CFU per milliliter was determined. The stock produced by ψ-CRIP had a titer of $6.20 \times 10^4 \pm 0.86 \times 10^4$ CFU/mL and the stock produced by TELCeB6-A had a titer of $7.12 \times 10^7 \pm 0.60 \times 10^7$.

Beta-galactosidase (β-gal) transduction assay. Five thousand NIH 3T3 murine fibroblasts in 100 μL of medium were plated per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, MA)[42]. The next day (19 to 26 h later), the medium was removed and 100 μL of the virus-containing solutions was added to each well. Two days after transduction, the medium was removed and the cells washed once with 100 μL of phosphate-buffered saline (PBS) containing 1mM $MgCl_2$. After removal of the wash solution, 50 μL of lysis buffer (PBS with 1mM $MgCl_2$ and 0.5% Igepal) were added to each well, and the plate incubated at

37°C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG was warmed to 37°C and then added to each well, and the plate incubated at 37°C for 5 to 60 min until a visible yellow color was obtained. The reactions were halted by the addition of 20 μL per well of stop buffer (1M Na_2CO_3). The optical density at 420 nm (OD_{420}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA) and the non-specific background at 650 nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.

Production of medium that contains free amphotropic envelope protein. TE671 cells, plated the previous day in a 10-cm dish (5 x 10^6 cells), were transfected with an expression plasmid (16 μg) for the amphotropic envelope glycoprotein (FB4070ASALF) using Lipofectamine 2000 (40 μL). Eight hours later the medium was replaced with fresh medium. Thirty-six hours after the start of the transfection the conditioned medium, which contained soluble envelope proteins shed from the surfaces of the transfected cells, was harvested, filtered (0.45μm) to remove cellular debris, and frozen (-80 °C) for later use.

Retrovirus purification. We compared three methods for purifying retrovirus: polymer complexation, ultracentrifugation, and low speed centrifugation. To purify retrovirus by polymer complexation, virus stock (0.4 to 1.5 mL per microcentrifuge tube) was brought to equal weight concentrations (80 μg/mL) of PB and CSC by adding, in immediate succession, appropriate volumes of aqueous stock solutions (20 mg/mL) of the polymers. The mixture was vortexed briefly, incubated for 20 min at 37°C, and then centrifuged to pellet the virus-polymer complexes (10000g, 5 min). The supernatant was removed and the pellet was resuspended to its original volume in fresh cell culture medium. To purify retrovirus by ultracentrifugation, virus stock (9 mL) was layered over

2 mL of 20% sucrose and then centrifuged for 90 min at 40000 rpm (200000g) at 4°C. Eight milliliters of the supernatant were collected (virus-free conditioned medium, VFCM), after which the rest of the supernatant was decanted and discarded, and the pellet was resuspended to its original volume in fresh medium. To purify retrovirus by low speed centrifugation, virus stock (0.4 to 1.5 mL per microcentrifuge tube) was centrifuged for 16 hrs at 6000g, 4°C. The supernatant was decanted and the pellet resuspended to its original volume in fresh medium.

ELISA for p30 and gp70. We used an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of virus capsid protein (p30) and viral envelope glycoprotein (gp70). ELISA plates (Nunc immuno Maxisorp 96-well plates, Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody or mouse anti-gp70 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to block non-specific binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, and then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 and gp70 were sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer or the goat polyclonal anti-gp70 diluted 1:300 in blocking buffer, and incubated for 1 h at 37°C. The horseradish peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer and then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed using hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm

(OD_{490}) was measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Protein assays. To visualize proteins in virus stocks, samples were boiled in Laemmli buffer and 5% β-mercaptoethanol (33.3 μL) for 5 min at 100°C, separated by size by SDS-PAGE (4-20% Tris-Glycine gel; Gradipore, Australia), and then silver-stained using GelCode SilverSNAP Stain (Pierce, Rockford, IL). Protein concentrations were determined with the Comassie Plus-200 Protein Assay Reagent (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Percent transduced assay. Cells were transduced in 96-well dishes as described above, grown to confluence, and trypsinized. Twenty to one hundred microliters of the trypsinized cells were brought to 1 mL in cell culture medium. Ten microliters of this solution was plated into a 10-cm dish. After 10 days, when macroscopic colonies had grown, the plates were washed once with PBS and fixed and stained for β-galactosidase as previously described [42]. The colonies were then counterstained with 1% rhodamine B, rinsed with water and air dried. Values for the percentage of *lacZ*$^+$ colonies at each point (blue colonies/total colonies x 100) are the averages of triplicate wells of the 96-well dish.

Determination of transgene copy number by real-time PCR. To compare the number of transgenes integrated per cell, clonally derived macroscopic colonies were obtained as described in the percent transduced assay. Individual colonies were isolated, expanded in 10-cm plates, and then their genomic DNA extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Real-time PCR was performed with an ABI PRISM 7700 sequence detector (Applied Biosystems) in a final volume of 30 μL. The

PCR mix contained SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), DNA (0.01 μg), and forward and reverse primers each brought to a final concentration of 30 μM. *LacZ*-specific primers were designed with Primer Express software (version 1.5) (Applied Biosystems). The sequences were as follows: forward primer 5'-TTCGCTACCTGGAGAGACGC-3', and reverse primer 5'-ATTTAGCGAAACCGCCAAGA-3'. The cycling conditions were 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative number of integrated transgenes per cell was determined using serial dilutions of lacZ amplicons ranging in concentration from $5x10^{-7}$ to 5 nM. Copy numbers were normalized to 18S DNA which was amplified under identical conditions using the forward primer 5'-CGCCGCTAGAGGTGAAATTC-3' and the reverse primer 5'-TTGGCAAATGCTTTCGCTC-3'. Values were reported as the number of copies per cell relative to the clone with the lowest number of integrated transgenes. Values are the averages of triplicate PCR tubes.

Data Analysis. Data are summarized as mean ± SD. Statistical analysis was performed using a one-way analysis of variance for repeated measurements of the same variable. The Tukey multiple comparison test was used to conduct pairwise comparisons between means. Differences at $p < 0.05$ were considered statistically significant.

5.4 Results

We have previously shown that the addition to virus stocks of Polybrene (PB), a cationic polymer, and chondroitin sulfate C (CSC), an anionic polymer, leads to the rapid formation of polyelectrolyte complexes that selectively incorporate retrovirus particles but few other proteins (refer to Chapter 2) [13]. Given the selectivity of this process, we

decided to test if polymer complexation could be used to purify virus stocks from inhibitors of transduction. We used stocks of *lacZ* amphotropic retrovirus produced by TELCeB6-A packaging cells because they appeared to contain high levels of inhibitory activity as evidenced by the highly non-linear relationship between the amount of virus used to transduce cells and the level of gene transfer. For example, when these viruses were used to transduce NIH 3T3 cells in a microplate assay the dose response curve had two components, a portion in which gene transfer increased in proportion to the dose of virus used (0 to 20 μL per well), and a portion in which gene transfer decreased with increasing doses of virus (above 20 μL per well) (Figure 5.1). To determine if the virus stocks contained inhibitory activity, we pelleted the virus particles from the stocks by ultracentrifugation, decanted the virus-free conditioned medium (VFCM) from the pelleted viruses, and tested its ability to inhibit transduction. We mixed 20 μL of amphotropic *lacZ* virus with a range of volumes of VFCM (0 to 80 μL), adjusted the final volume to 100 μL with fresh medium, and then used the solution to transduce NIH 3T3 cells. Transduction was inhibited in a dose-dependent manner by increasing concentrations of VFCM (Figure 5.2) which confirmed that the virus stocks contained inhibitors of transduction.

To determine if the inhibitory activity was specific for amphotropic retroviruses, we transduced NIH 3T3 cells with 80 μL of VFCM or fresh medium, and 20 μL of one of the following virus stocks: amphotropic virus produced by TELCeB6-A packaging cells, amphotropic virus produced by ψ-CRIP packaging cells, or ecotropic virus produced by CRE-BAG packaging cells. VFCM inhibited transduction by 7-fold or more when amphotropic virus was used, but had no significant effect on transduction when ecotropic virus was used (Figure 5.3). These results suggest that the inhibitory activity is specific for amphotropic retrovirus and may consist of free envelope glycoproteins (gp70) that

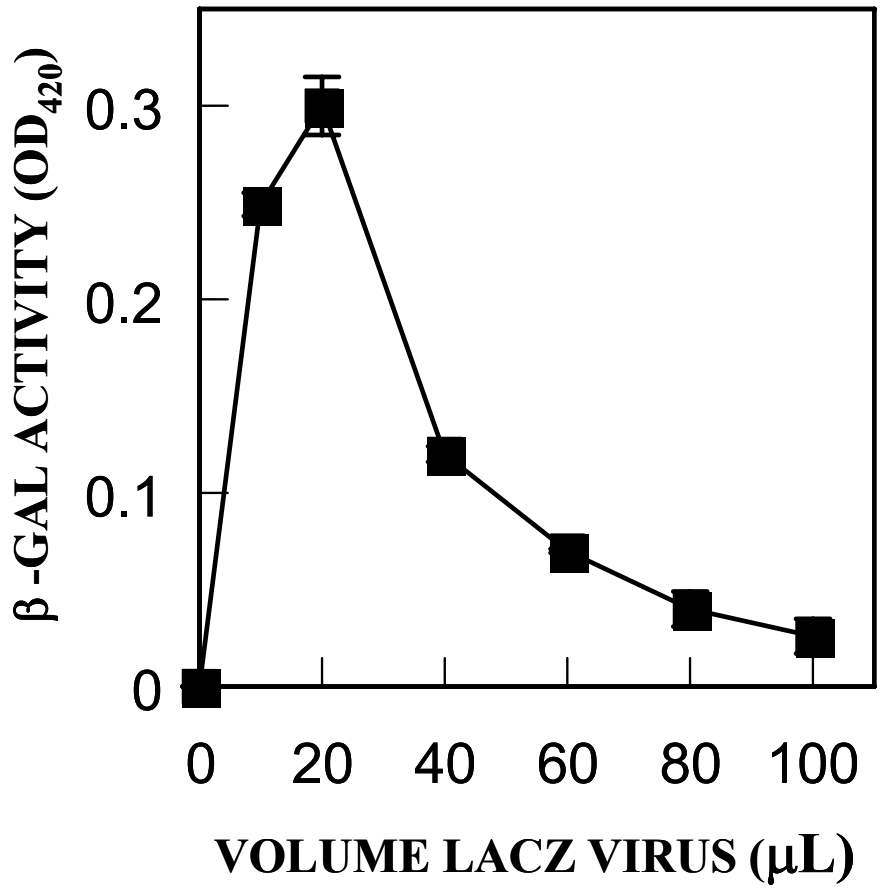

Figure 5.1 Retrovirus produced by TELCeB6-A cells contains substances that inhibit retrovirus transduction. NIH 3T3 cells were plated in a 96-well dish (5,000 cells per well) and the next day transduced with 100 μL of medium than contained various volumes of amphotropic *lacZ* retrovirus produced from the TELCeB6-A cell line, 8 μg/mL PB and fresh medium. Two days later the amount of β-galactosidase (β-gal) activity in lysates of the cells, which is proportional to the level of gene transfer, was quantified using the β-gal transduction assay. Each point represents the mean ± standard deviation of triplicate samples.

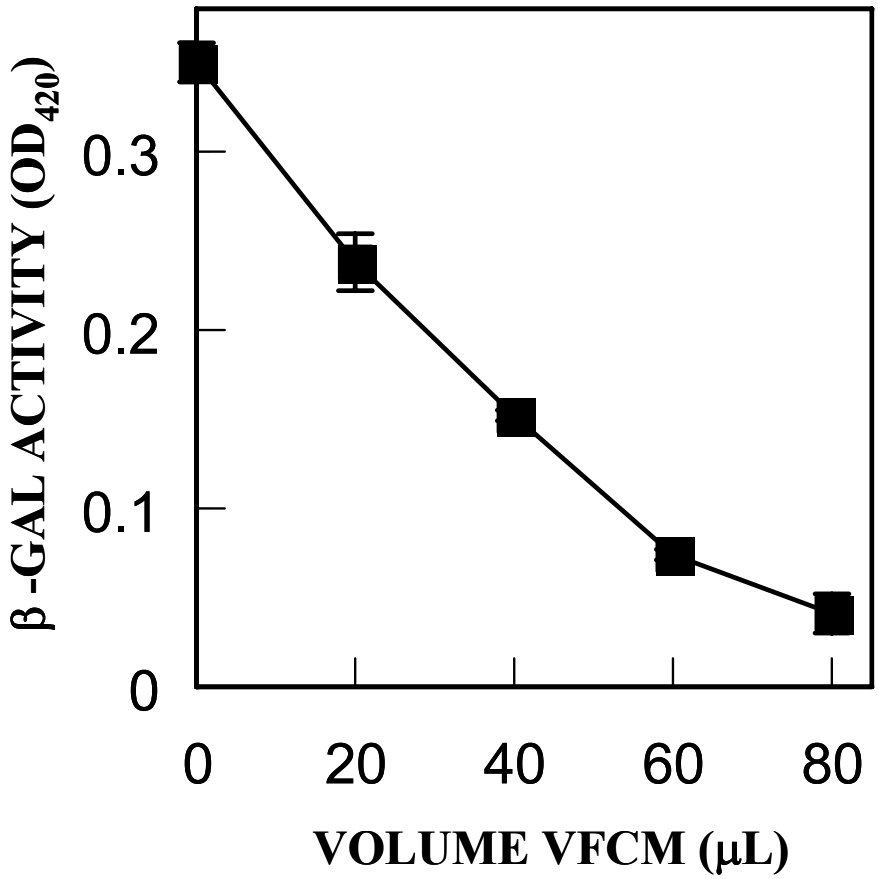

Figure 5.2 The conditioned medium produced TELCeB6-A cells contains substances that inhibit retrovirus transduction. NIH 3T3 cells were plated in a 96-well dish (5,000 cells per well) and the next day transduced with 100 μL of medium that contained various volumes of virus-free medium conditioned by TELCeB6-A cells (VFCM), 20 μL of amphotropic *lacZ* virus stock produced by TELCeB6-A cells, 8 μg/mL of PB, and fresh medium. Two days later the wells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate samples.

are not associated with virus particles but which competitively bind to cell surface amphotropic receptors, thereby inhibiting amphotropic retrovirus transduction.

Given that in a previous study we had shown that PB-CSC polymer complexes efficiently incorporate intact virus particles, we reasoned that polymer complexation might be useful for purifying intact virus particles from inhibitory free envelope proteins if the free envelope proteins were incorporated into the complexes less efficiently than intact viruses. To determine the extent to which free envelope proteins are incorporated into PB-CSC complexes, we generated medium that contained free amphotropic envelope proteins by transiently transfecting TE671 cells, the parent cell line of TELCeB6-A packaging cells, with an expression plasmid for the amphotropic envelope protein. Cell culture medium that was conditioned by these cells and which contained free amphotropic envelope proteins, was brought to equal weight concentrations of PB (80 μg/mL) and CSC (80 μg/mL), incubated for 20 minutes at 37°C to allow the charged polymers to form polyelectrolyte complexes, and then centrifuged (10000g) for 5 minutes to pellet the complexes. We decanted the supernatant from the pelleted complexes, resuspended the complexes to their original volume in fresh cell culture medium, and then quantified by ELISA the amount of free envelope protein in the supernatant and resuspended pellets. The concentration of free envelope protein in the original conditioned medium and in the supernatant was not signficantly different ($p < 0.05$) (Figure 5.4). Similar results were obtained with VFCM that contained free envelope proteins (data not shown). These results show that, unlike intact virus particles, free retrovirus envelope proteins are not efficiently incorporated into PB-CSC polymer complexes.

To examine the extent to which PB-CSC complexes incorporate other proteins, we repeated the polymer complexation and resuspension procedure with amphotropic virus stocks. We diluted samples of the supernatant and resuspended pellets in PBS,

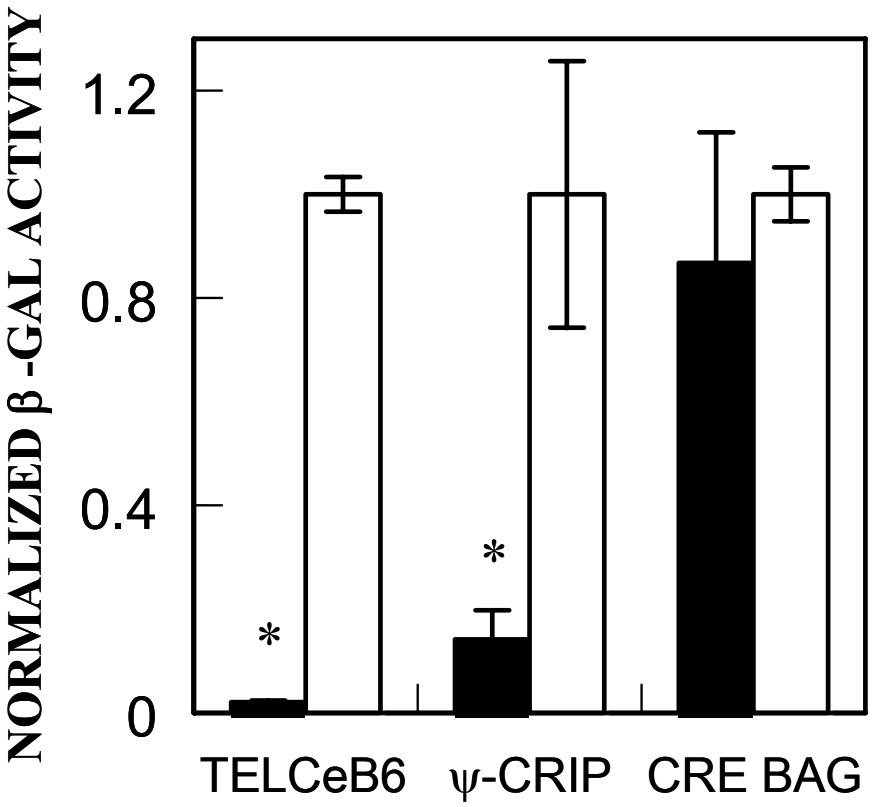

Figure 5.3 The conditioned medium produced TELCeB6-A cells contains substances that inhibit amphotropic, but not ecotropic, retrovirus transduction. NIH 3T3 cells were plated in a 96-well dish (5,000 cells per well) and the next day transduced with 100 μL of medium that contained 80 μL of VFCM (black bars) or fresh medium (white bars), 8 μg/mL of PB, and 20 μL of either amphotropic *lacZ* virus produced from TELCeB6-A cells, amphotropic lacZ virus produced from ψ-CRIP cells, or ecotropic *lacZ* retrovirus produced from CRE BAG cells. Two days later the wells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate samples. Statistically significant differences ($p \leq 0.05$) from virus stocks that were diluted in fresh medium are denoted with an asterisk.

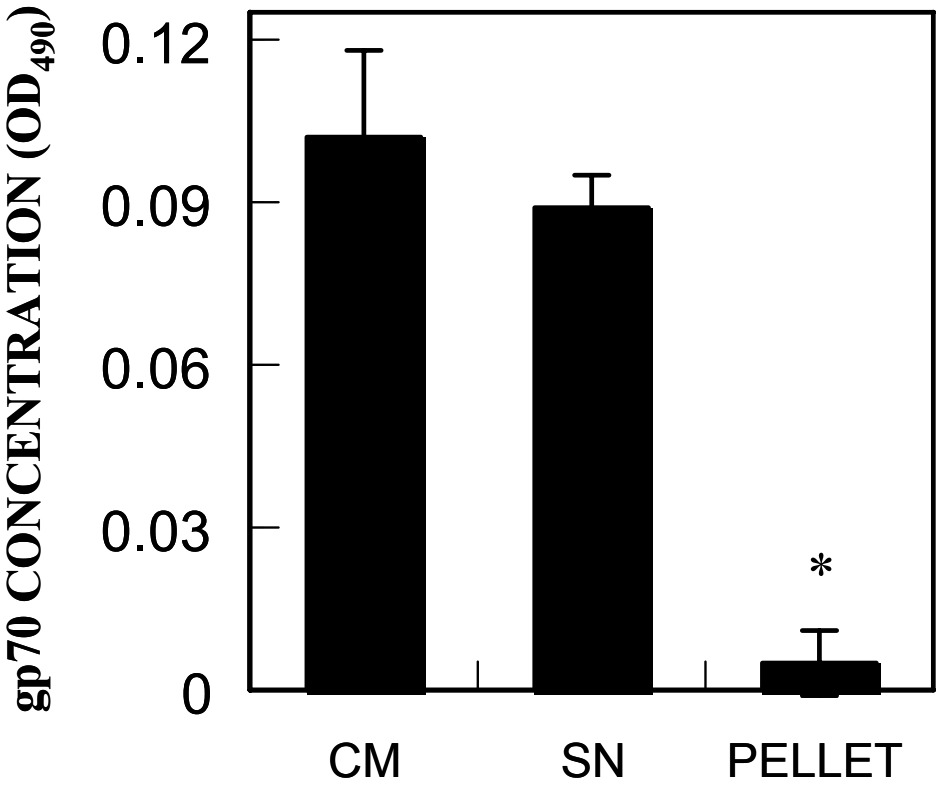

Figure 5.4 Complexes of CSC and PB do not incorporate amphotropic envelope protein. Conditioned medium that contained free amphotropic envelope protein, generated by incubation of fresh cell culture medium for 28 h with confluent cultures of TE671 cells transiently transfected with an expression plasmid for the amphotropic envelope protein (FB4070ASALF), were brought to 80 μg/mL of CSC and 80 μg/mL PB, incubated 20 min at 37°C to allow complexes of CSC and PB to form, and then centrifuged to pellet the complexes. The pellet was resuspended in fresh medium to its original volume. The concentration of amphotropic envelope protein in the conditioned medium (CM), supernatant (SN), and resuspended pellet (PELLET) was determined with an ELISA for gp70 (OD_{490}). Each point shows the mean ± standard deviation of three replicates. Statistically significant differences ($p \leq 0.05$) from CM are denoted by an asterisk.

separated them by size by polyacrylamide gel electrophoresis, and then visualized the proteins in the gel by silver staining. As controls, we also analyzed samples of neat virus stock, and virus stock that contained PB and CSC but which had not been centrifuged. The supernatant contained large quantities of numerous proteins and was indistinguishable from the virus stocks that had not been centrifuged (Figure 5.5, lanes 1-3). In contrast, only small quantities of high molecular weight proteins (> 250 kDa) were detected in the resuspended pellets (Figure 5.5, lane 4), even when added at concentrations 10-fold higher than that of the other solutions (Figure 5.5, lane 5). We also quantified the percentage of total protein and viral capsid protein (p30) that was incorporated into the complexes. We found that the complexes captured 72% of the virus particles, but only 0.36% of the total protein that was initially present in the virus stock.

These data show that PB-CSC complexes capture very few non virus-associated proteins. Nevertheless, it remained a possibility that the high molecular weight proteins associated with the complexes inhibit transduction, or that the complexes contain inhibitors of transduction that were not detected by our assays. To address this possibility, we directly quantified the level of inhibitory activity that was present in the complexes. We formed polymer complexes in VFCM, pelleted the complexes by centrifugation, and decanted the VFCM supernatant. In parallel, we performed the same procedure with fresh medium. We then resuspended both sets of complexes to their original volume in the supernatant that was decanted from the complexes formed in VFCM, or in the supernatant that was decanted from the complexes formed in fresh medium. To measure their inhibitory activity, we mixed 80 μL of these solutions with 20 μL of amphotropic *lacZ* virus and used them to transduce NIH 3T3 cells. We found that complexes formed in VFCM and resuspended in fresh medium did not inhibit gene

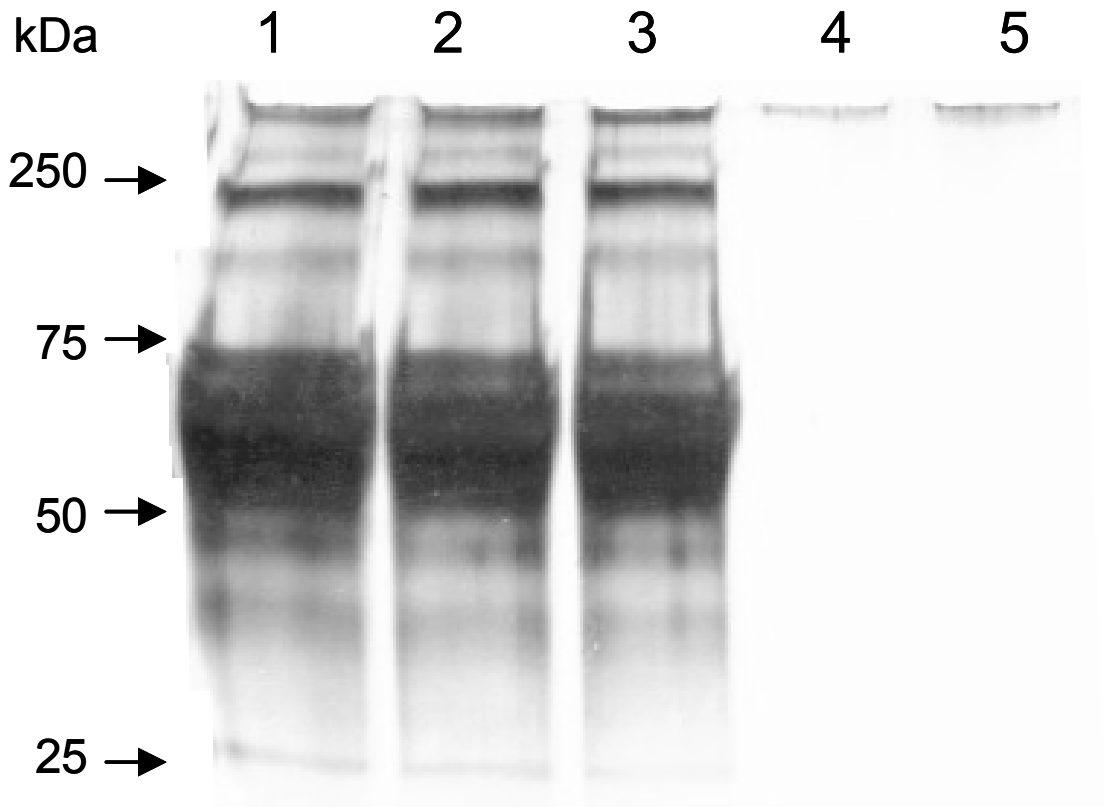

Figure 5.5 Complexes of CSC and PB do not incorporate most proteins. *LacZ* amphotropic virus stock produced by TELCeB6-A cells was brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, and then the complexes pelleted by centrifugation and resuspended to their original volume in PBS. The proteins in 1.7 μL of virus stock (lane 1), virus stock brought to 80 μg/mL each of PB and CSC (lane 2), supernatant (lane 3), and resuspended pellet (land 4), and 17 μL of resuspended pellet (lane 5) were separated by size under reducing conditions by SDS-PAGE (4-20% tris-glycine) and then visualized by silver-staining. The mobilities of molecular mass markers are indicated on the left.

transfer. This shows that PB-CSC complexes do not incorporate detectable levels of inhibitory activity (Figure 5.6).

These results suggest that polymer complexation could be useful for purifying retroviruses from inhibitors of transduction. To test this we formed polymer complexes in amphotropic virus stocks produced by TELCeB6-A and Ψ-CRIP cells, pelleted them by centrifugation, decanted the supernatant, resuspended the complexes to their original volume in fresh cell culture medium, or as a control in the decanted supernatant, and used them to transduce NIH 3T3 cells. We compared the levels of transduction achieved by these processed virus stocks to the levels of transduction achieved by virus stocks that were not processed with the polymer complexes (Figure 5.7). The level of enhancement ranged from 21 to 186-fold depending on which virus harvest was used, presumably because the level of inhibitory activity within the virus stocks varied from harvest to harvest (Figure 5.8). In contrast, complexation and resuspension in fresh medium of viruses produced by Ψ-CRIP cells did not enhance transduction (Figure 5.7), most likely because Ψ-CRIP virus stocks contain much lower levels of inhibitory activity [6].

To determine if this virus processing strategy could be used to increase gene transfer to other cell types, we formed polymer complexes with amphotropic virus stocks produced by TELCeB6-A cells, pelleted the virus-polymer complexes, resuspended them to their original volume in fresh medium, and used them to transduce rat intestinal epithelial (RIE), human adenocarcinoma (HeLa), and NIH 3T3 cells. In parallel, we transduced cells with virus-polymer complexes that were not pelleted or resuspended in fresh medium. We found that virus stocks that had been pelleted and resuspended in fresh medium transduced these cell lines 6 to 19-fold more efficiently than virus stocks that were not pelleted and resuspended in fresh medium (Figure 5.9).

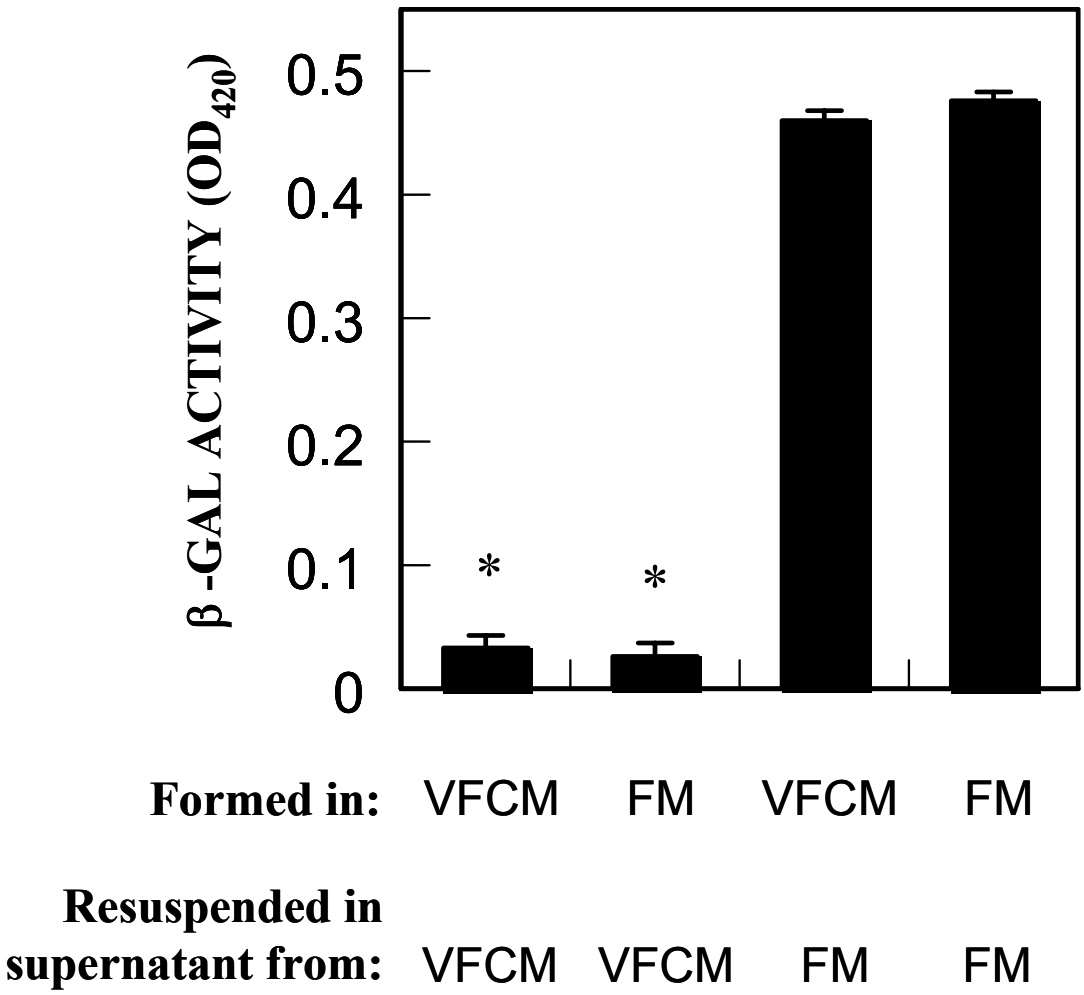

Figure 5.6 Complexes of CSC and PB do not incorporate substances that inhibit amphotropic retrovirus transduction. VFCM, which contains substances that inhibit amphotropic retrovirus transduction, was brought to 80 μg/mL of CSC and 80 μg/mL PB, incubated 20 min at 37°C to allow complexes of CSC and PB to form, and then centrifuged to pellet the complexes. In parallel we conducted the same procedure with fresh medium (FM). The pellets from VFCM and FM were resuspended to their original volumes in supernatant that was decanted from the complexes formed in VFCM or in the supernatant that was decanted from the complexes formed in FM. Twenty microliters of *LacZ* amphotropic virus stock produced by TELCeB6-A cells was brought to 100 μL with each of the resuspended pellets and to 8 μg/mL of PB, and then used to transduce NIH 3T3 cells. Two days later the cells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate samples. Statistically significant differences ($p \leq 0.05$) from the complexes formed in FM and resuspended in the supernatant that was decanted from the complexes formed in FM are denoted by an asterisk.

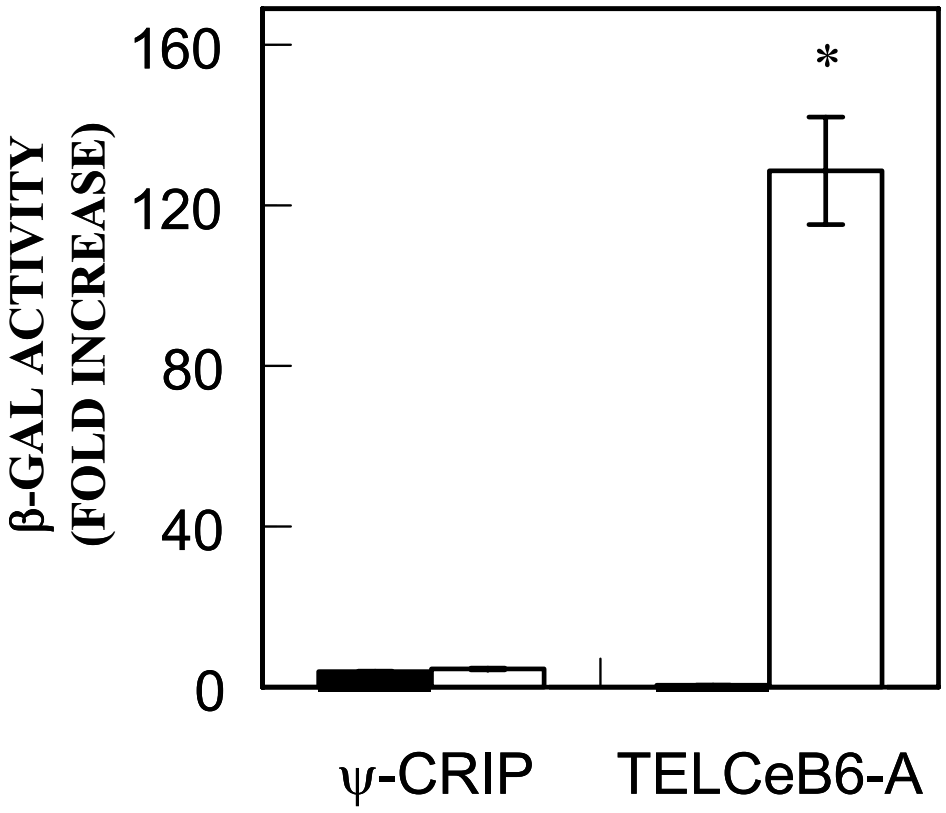

Figure 5.7 Complexation of retrovirus with CSC and PB purifies them from inhibitors and substantially increases the efficiency of retrovirus transduction. *LacZ* amphotropic virus stocks produced by TELCeB6-A and ψ-CRIP cells were brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, and then centrifuged to pellet the virus-polymer complexes. The supernatant was decanted and the pelleted complexes were resuspended to their original volume in fresh medium (white bars) or in the supernatant (black bars), brought to 8 μg/mL of PB, and used to transduce NIH 3T3 cells plated the previous day in a 96-well dish (5,000 cells per well). Two days later the wells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate samples. Statistically significant differences ($p \leq 0.05$) from complexes that were resuspended in supernatant are denoted with an asterisk.

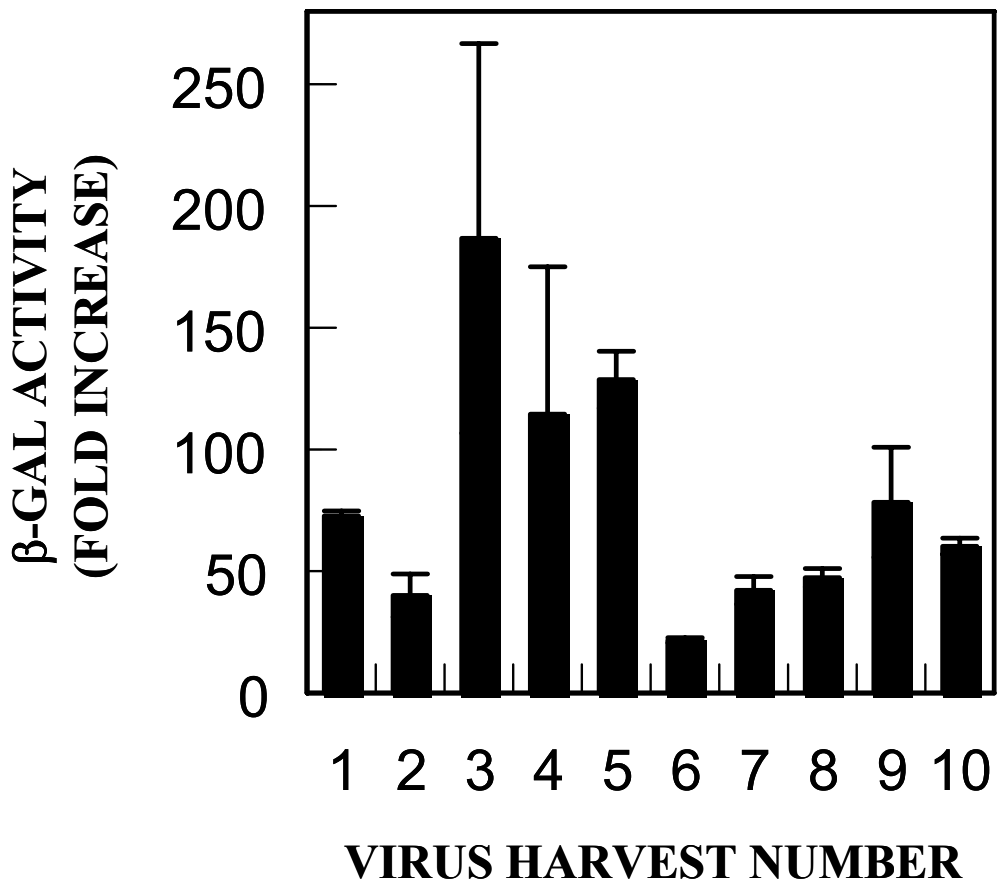

Figure 5.8 The level of enhancement in gene transfer varies depending on the virus harvest. Ten different harvests of *LacZ* amphotropic virus stock produced by TELCeB6-A cells were brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, and then centrifuged to pellet the virus-polymer complexes. The supernatant was decanted and the pelleted complexes were resuspended to their original volume in fresh medium, brought to 8 μg/mL of PB, and used to transduce NIH 3T3 cells plated the previous day in a 96-well dish (5,000 cells per well). Two days later the wells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate samples.

Next we wanted to determine the relationship between the level of gene transfer and the concentration of polymer-purified retrovirus used to transduce cells. We brought a range of volumes of polymer-purified virus (10 to 100 μL) to 100 μL with fresh medium and then used the solutions to transduce NIH 3T3 cells. When low doses of virus (10 to 20 μL per well) were used a linear relationship between virus dose and gene transfer was observed, whereas when higher doses (above 20 μL) of virus were used transduction reached a plateau and remained virtually constant (Figure 5.10). A similar trend was observed when we measured the percentage of cells that had been transduced (Figure 5.11).

We wondered if the plateau in beta-galactosidase activity was because the transduced cells contained the same number of integrated transgenes and therefore represented a maximum in the level of gene transfer that could be attained, or if the cells differed in the number of integrated transgenes but were unable, above a certain threshold number of integrated transgenes, to synthesize more beta-galactosidase. To distinguish between these two possibilities, we examined the relationship between virus concentration and the number of integrated transgenes per cell. We transduced NIH-3T3 cells with a range of concentrations of polymer-purified virus, isolated and expanded three or more clonal cell lines from each of these mixed populations of transduced cells, and then used real-time PCR to quantify the relative number of integrated transgenes per cell in each clonal cell line. We found that the number of integrated transgenes reached a maximum when 40 μL per well or more of purified virus was used to transduce cells (Figure 5.12).

We wondered if the plateau in transduction levels was due to the presence of residual inhibitory activity, an excess of polymers, or if the cells were becoming saturated with virus particles. We reasoned that if we reduced the concentration of virus

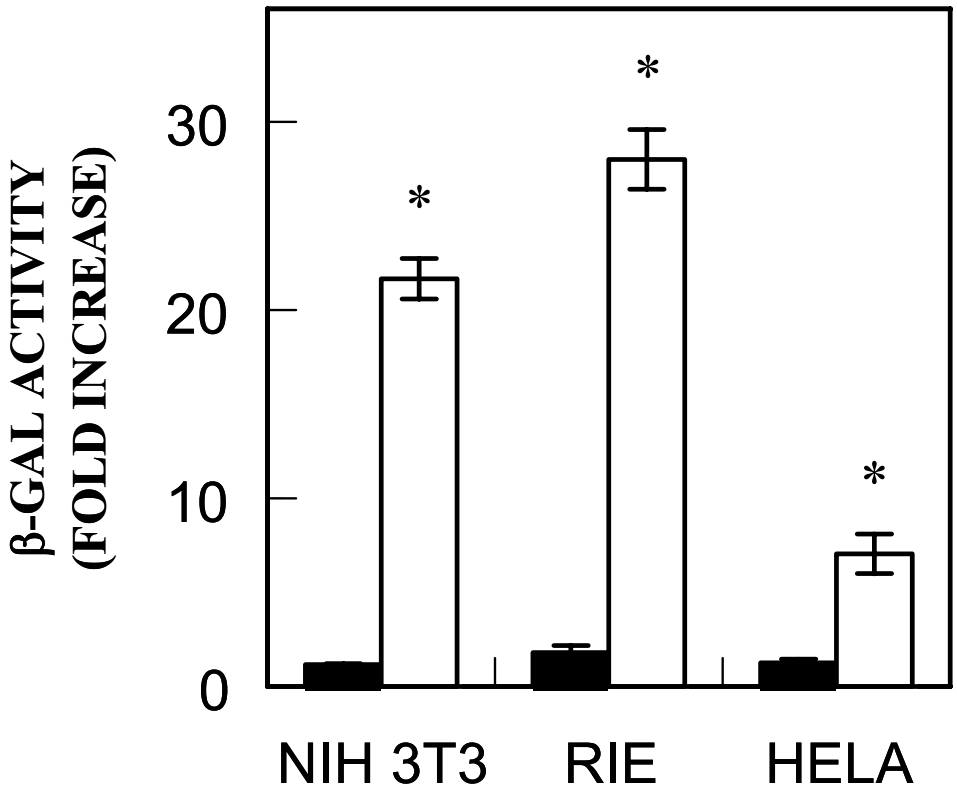

Figure 5.9 Complexation of retrovirus with CSC and PB increases the efficiency of increase gene transfer to other cell types. *LacZ* amphotropic virus stock produced by TELCeB6-A cells was brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, and then centrifuged to pellet the virus-polymer complexes. The supernatant was decanted and the pelleted complexes were resuspended to their original volume in fresh medium (white bars) or in the supernatant (black bars), brought to 8 μg/mL of PB, and used to transduce NIH 3T3, rat intestinal epithelial (RIE), and HeLa (HELA) cells plated the previous day in a 96-well dish (5,000 cells per well). Two days later the wells were assayed for β-gal activity. Values are fold-increases in gene transfer with respect to transduction with neat virus stocks containing 8 μg/mL of PB. Each point represents the mean ± standard deviation of triplicate samples. Statistically significant differences ($p \leq 0.05$) from complexes that were resuspended in supernatant are denoted with an asterisk.

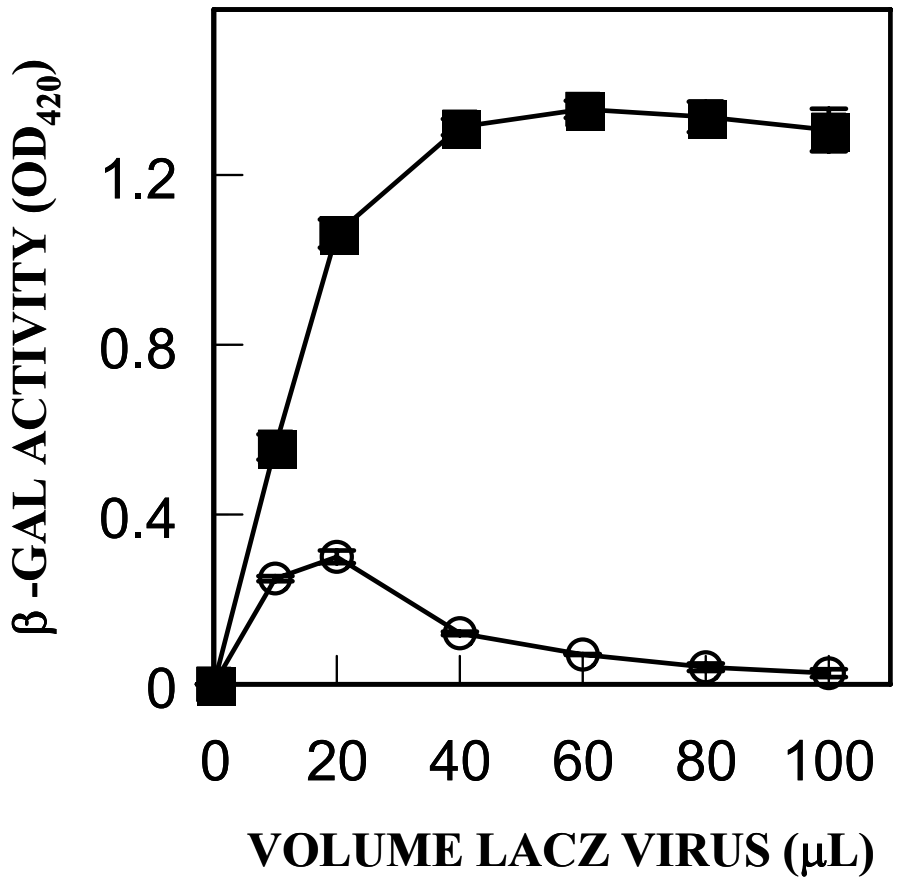

Figure 5.10 Dose-response curves for neat and purified amphotropic *lacZ* retrovirus produced by TELCeB6-A cells. *LacZ* amphotropic virus stock produced by TELCeB6-A cells was brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, centrifuged to pellet the virus-polymer complexes, after which the pelleted complexes were resuspended to their original volume in fresh medium. A range of volumes (0 to 100 μL) of the original virus stock (○) or the resuspended complexes (■) were brought to 100 μL with fresh medium and to 8 μg/mL PB, and then used to transduce NIH 3T3 plated the previous day in a 96-well dish (5,000 cells per well). Two days later the wells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate wells of the 96-well dish.

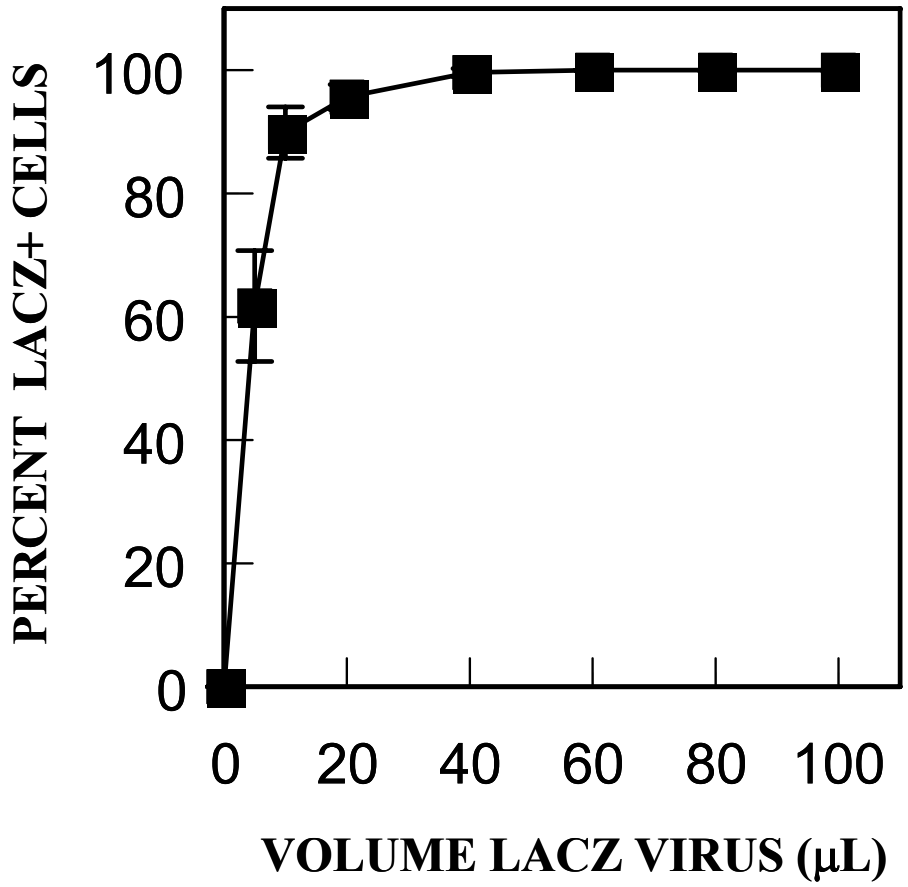

Figure 5.11 Percentage transduction with purified amphotropic *lacZ* retrovirus produced by TELCeB6-A cells. *LacZ* amphotropic virus stock produced by TELCeB6-A cells was brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, centrifuged to pellet the virus-polymer complexes, after which the pelleted complexes were resuspended to their original volume in fresh medium. A range of volumes (0 to 100 μL) of the resuspended complexes were brought to 100 μL with fresh medium and to 8 μg/mL PB, and then used to transduce NIH 3T3 plated the previous day in a 96-well dish (5,000 cells per well). Two days later the cells were trypsinized, diluted, and plated onto a 10-cm dish. After 10 days of growth, macroscopic colonies were fixed, stained, and the percentage of *lacZ*$^+$ colonies calculated. Each point represents the mean ± standard deviation of triplicate wells of the 96-well dish.

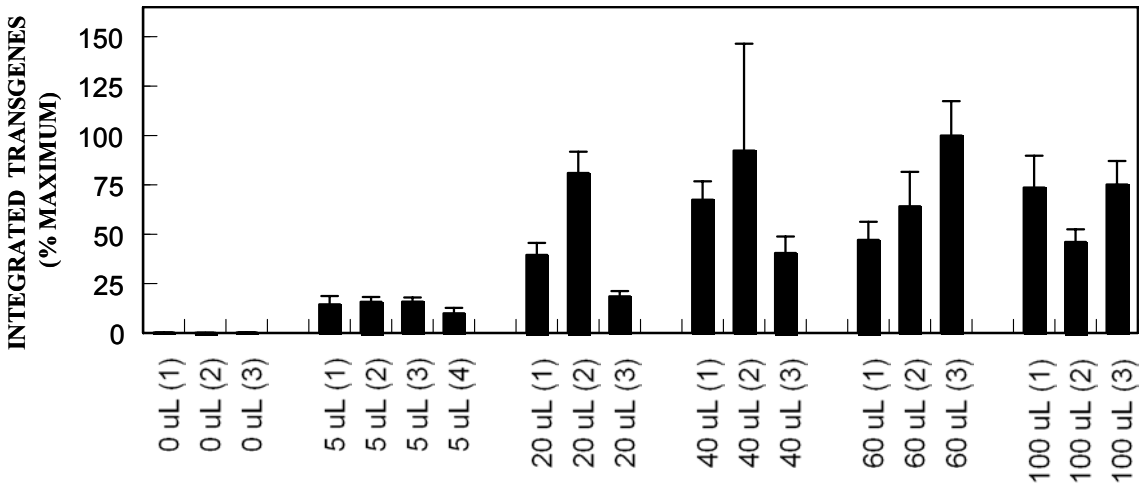

Figure 5.12 The number of integrated transgenes reaches a maximum when high doses of purified retrovirus are used to transduce NIH 3T3 cells. *LacZ* amphotropic virus stock produced by TELCeB6-A cells was brought to 80 μg/mL CSC and 80 μg/mL PB, incubated for 20 min at 37°C, centrifuged to pellet the virus-polymer complexes, and then the pelleted complexes were resuspended to their original volume in fresh medium. A range of volumes (5 to 100 μL) of the resuspended complexes were brought to 100 μL with fresh medium and to 8 μg/mL PB, and then used to transduce NIH 3T3 plated the previous day in a 96-well dish (5,000 cells per well). Two days after transduction, cells from each well were trypsinized, diluted in fresh medium and plated at clonal density in 10-cm plates. Ten days later, three or four individual colonies from each plate were selected and expanded (different colonies from the same plate are denoted by the number in parenthesis). The genomic DNA of individual colonies was extracted and the relative number of integrated transgenes quantified by Real Time PCR. Values are the number of integrated transgenes as a percentage of the maximum number detected. Each point represents the mean ± standard deviation of triplicate measurements.

while keeping the composition of the medium in which the viruses were suspended the same, we could distinguish between saturation of the cells with virus and other possible causes of the maximum in transduction. Therefore, we diluted amphotropic virus stocks produced by TELCeB6-A cells five-fold with VFCM or fresh medium, formed polymer complexes within them, pelleted the complexes by centrifugation, resuspended the pellet to its original volume in fresh medium and used them to transduce NIH-3T3 cells. The dose response curves were linear and virtually identical, regardless of whether the virus was originally diluted in fresh medium or conditioned medium (Figure 5.13). These results suggest that the plateau in transduction is not due to the presence of inhibitors or to an excess of charged polymers in the purified virus stocks, and may be due to saturation of the cells with virus.

Finally, we compared transduction by retrovirus purified by polymer complexation to transduction by retrovirus purified by ultracentrifugation and low speed centrifugation. To purify virus stocks by ultracentrifugation, we centrifuged them for 90 min at 200000g over a sucrose cushion, and then resuspended the pelleted viruses to their original volume in fresh medium. To purify virus stocks by low speed centrifugation, we centrifuged virus stocks for 16 hours at 6000g, and then resuspended the pelleted viruses to their original volume in fresh medium. Interestingly, transduction by polymer-purified virus was 2-fold more efficient than virus purified by ultracentrifugation and 4-fold more efficient than virus purified by low speed overnight centrifugation (Table 5.1). These differences in transduction did not appear to be due to differences in virus recovery since the amount of virus recovered by the three purification methods varied by no more than 1.4-fold as quantified by virus titer or p30 ELISA.

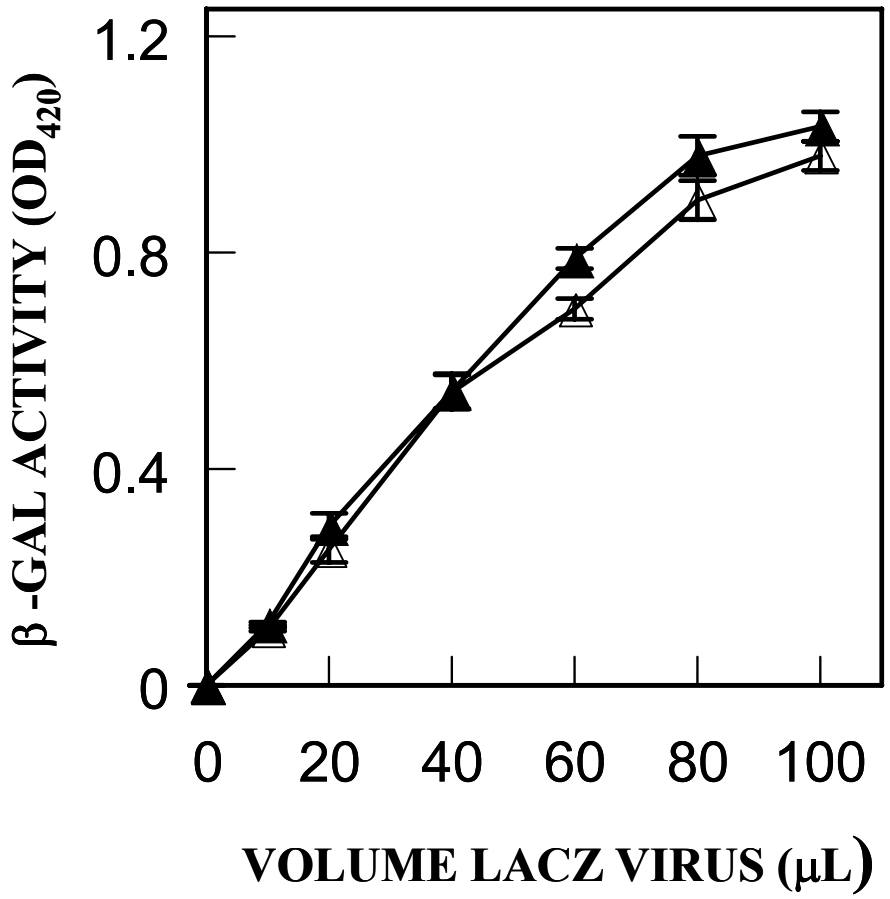

Figure 5.13 Transduction by virus purified by complexation with polymers is not limited by the polymers or by the presence of residual inhibitory activity. Twenty microliters of *LacZ* amphotropic virus stock produced by TELCeB6-A cells was mixed with 80 μL of fresh medium (△) or virus-free conditioned medium (▲), brought to 80 μg/mL of CSC and 80 μg/mL PB and incubated 20 min at 37°C to allow complexes of virus, CSC, and PB to form. The complexes were pelleted by centrifugation, and then resuspended to their original volume in fresh medium. A range of volumes (10 to 100 μL) of the resuspended virus-polymer complexes were brought to 100 μL with fresh medium and to 8 μg/mL Polybrene, and then used to transduce NIH 3T3 plated the previous day in a 96-well dish (5,000 cells per well). Two days later the wells were assayed for β-gal activity. Each point represents the mean ± standard deviation of triplicate wells of the 96-well dish.

Table 5.1 Purification of retrovirus by ultracentrifugation, low-speed centrifugation, and complexation with CSC and PB[a]

Purification method	p30 Capsid protein (% recovery)[b]	Virus titer (% recovery)[b]	Fold-enhancement[c]
Ultracentrifugation	83 ± 19	62 ± 21	26 ± 4.0*
Low-speed centrifugation	91 ± 22	59 ± 15	12 ± 1.9*
Polymer complexation	83 ± 16	81 ± 20	50 ± 7.8

[a]*LacZ* virus produced by TELCeB6-A cells was purified by three different methods: centrifugation for 90 min at 200000g over a sucrose cushion (ultracentrifugation), centrifugation for 16 hours at 6000g (low-speed centrifugation), or by complexation with 80 μg/mL each of PB and CSC followed by centrifugation for 5 min at 10000g (polymer complexation). Centrifuged virus was resuspended to its original volume in fresh medium and then analyzed for concentration of p30, virus titer, and transduction efficiency as described in the Materials and Methods.

[b]% recovery was defined as the ratio, multiplied by 100, of the p30 concentration (or virus titer) of virus that had been centrifuged and resuspended to its original volume to the p30 concentration (or virus titer) of neat virus stocks. Values are the means ± SD for at least three replicates.

[c] Fold-enhancement of transduction was defined as the ratio of the beta-galactosidase activity in the lysates of cells transduced with purified virus to the beta-galactosidase activity in the lysates of cells transduced with neat virus stocks. Values are the means ± SD for at least three replicates.

*Statistically significant differences ($p \leq 0.05$) from virus stocks that were purified by polymer-complexation were denoted with an asterisk.

5.5 Discussion

We have previously shown that retroviruses can be rapidly concentrated by complexation with the anionic polymer chondroitin sulfate C (CSC) and the cationic polymer Polybrene (PB) (refer to Chapter 2) [13]. Given the selectivity with which retroviruses are incorporated into complexes of these polymers, we hypothesized that virus-polymer complexation could be used to purify viruses from inhibitors of transduction. To test this hypothesis, we formed virus-polymer complexes in amphotropic retrovirus stocks produced by TELCeB6-A cells, pelleted the complexes to separate them from the conditioned medium, and then examined the effect of this separation process on the level of transduction. We found that polymer complexation was an effective means to purify retrovirus. More than 70% of the virus particles, but less than 0.4% of all other proteins, were incorporated into the complexes and the complexes contained no detectable inhibitory activity. Viruses that were purified by complexation and resuspension in fresh medium transduced cells an average of 80-fold more efficiently than viruses that were not purified. In addition to substantially enhancing the level of gene transfer, virus purification also significantly altered the dose response of transduction. When virus that had not been purified was used, the relationship between transduction and virus concentration was highly non-linear. In contrast, when purified virus was used, transduction increased monotonically and was linearly proportional to virus concentration, except when high doses of virus were used. Our results show the benefits of purifying retrovirus prior to using them to transduce cells: virus purification increases the maximum number of genes that can be transferred, reduces the amount of virus required to achieve a given level of gene transfer, and reduces uncertainties about the relationship between the amount of virus used and the number of genes transferred.

Given that the purified virus contained no detectable levels of inhibitory activity, we were surprised to find that gene transfer reached a maximum plateau level when high doses of purified virus were used. The plateau in gene transfer was eliminated when we diluted the virus five-fold while keeping the composition of the medium constant, which suggested that the plateau was not due to the presence of residual inhibitory activity or an excess of polymers. Rather, we speculate that the cells were saturated with virus. We estimate, based on the virus titer and a previously described mathematical model that accounts for several key parameters that govern retrovirus adsorption [15], that the virus stocks contained about 10^9 virus particles/mL. One hundred microliters of this virus, complexed with polymers, were used to transduce about 20,000 cells per well in a 96-well dish. From a previous study, we estimate that about 80% of these virus-polymer complexes reached the surface of the tissue culture plate within the first 8 hours of transduction (refer to Chapter 3) [35]. Since the cells occupied about 10% of the surface area of the tissue culture dish, we estimate that about 400 virus particles were delivered per cell. Assuming that retroviruses each contain about 200-400 envelope proteins [43, 44], we estimate that about 10^5 envelope proteins were delivered per cell. Interestingly, NIH 3T3 cells express about 10^5 amphotropic receptors per cell [45]. Since retroviruses must bind to multiple receptors at the same time in order to transduce a cell [46, 47], it is likely that only a fraction of the receptors would need to be blocked or otherwise inactivated in order to render a cell resistant to infection. These approximate calculations suggest that it is possible the plateau we observed in the dose response curve of polymer purified virus was due to saturation of the cellular receptors. More experimental studies are needed to directly test this hypothesis.

We did not isolate the inhibitory activity but our data suggests that it is free amphotropic envelope protein (i.e., envelope proteins that are not associated with a virus

particle) since amphotropic, but not ecotropic, retrovirus transduction was blocked. Several previous studies have shown that free envelope proteins are present in retrovirus stocks and inhibit transduction [9, 10, 45, 48, 49], but the magnitude of inhibition in our system was unusually high. Neat virus stocks transferred 10-fold fewer genes than the same virus stock that was diluted 10-fold in fresh medium. Slingsby et al previously reported similar levels of inhibition due to the presence of amphotropic envelope protein in virus stocks produced by FLYA13 packaging cells [9]. In contrast to our findings, however, they found only low levels of inhibitory activity in virus produced by TEFLYA packaging cells, cells that are derived from the same parental cell line (TE671 cells) that was used to construct the packaging cell lines we used for our experiments (i.e., TELCeB6-A cells). Most likely, the level of free envelope protein that is present in virus stocks can vary widely between individual packaging cell lines even when they are derived from the same parental cell line. Indeed, we have recently constructed several clonal amphotropic retrovirus packaging cell lines from TELCeB6 cells and found that virus stocks with similar titers (within 2-fold of each other) can vary by 10-fold or more in their levels of soluble envelope protein (data not shown, refer to Chapter 6).

We find it interesting that only recently, with the advent of high titer ($>10^7$ CFU/mL) virus stocks, have investigators begun to observe this magnitude of inhibition. Although we can only speculate, we wonder if high titer virus stocks are more likely than low titer stocks to contain large numbers of inhibitory free envelope proteins. Since the expression cassettes for the genes that encode the structural proteins needed to form a virus particle are not linked to the expression cassette for the envelope proteins, it is not immediately clear why packaging cell lines that generate more virus particles would necessarily generate more soluble envelope proteins. Perhaps the process of selecting and isolating clonal cell lines that produce high titer virus stocks leads to the de facto

selection of cell lines that express high levels of envelope protein. Bachrach et al showed that virus titers increase in proportion to the amount of envelope protein that is incorporated into the virus particles, at least up to a minimum threshold level, and the amount of envelope protein incorporated into virus particles is in turn proportional to the level of envelope expression within the cell [46]. Therefore, a packaging cell line that produces large numbers of virus particles would presumably also have to produce a proportionally higher level of envelope virus protein in order to generate a high titer virus stock. It is also possible that soluble envelope proteins are shed from the virus particles themselves, rather than from the cells that produce the virus. Whether or not envelope proteins are shed from mature retrovirus particles, and to what extent, is somewhat controversial. Early studies suggested that the shedding of envelope proteins was a common phenomenon of retroviruses and lentiviruses [50-52], but more recent quantitative studies of the protein content of retroviruses and lentiviruses have demonstrated that envelope proteins are more stably associated with virus particles than previously thought and that lentiviruses shed few, if any, envelope proteins [53, 54]. Even if significant amounts of envelope proteins are shed from retrovirus particles, a very high concentration of virus would likely be required to generate a sufficient number of soluble envelope proteins to inhibit transduction. Battini et al showed that about 0.5 to 1 nM of a purified fragment of the amphotropic envelope protein was sufficient to significantly inhibit transduction [45]. McKeating et al found that similar concentrations of recombinant gp120 significantly inhibited HIV-1 infection [50]. If we assume, based on recent studies with HIV and SIV, that retroviruses shed about half of their envelope proteins as they age, then a virus stock would need to contain $2x10^9$ particles/mL or more in order to realistically generate enough free envelope protein to significantly inhibit transduction [51,55].

Although more work is needed to fully understand the origin of free envelope proteins, it is clear that their presence in retrovirus stocks is a significant barrier to achieving efficient, predictable, and reproducible levels of gene transfer. In this study we showed that polymer complexation is an effective means to purify retrovirus from free envelope protein. An important benefit of using polymer complexation to purify virus is that retroviruses are incorporated into the complexes regardless of which envelope protein they are pseudotyped with [13], and even when they are not pseudotyped with any envelope protein (refer to Chapter 2). As a result, unlike some other purification methods, viruses do not need to be pseudotyped with a specific envelope protein, such as VSV-G or an envelope protein into which special sequences have been inserted, in order to purify them [33, 56].

The mechanism by which retroviruses are selectively incorporated into complexes of PB and CSC is not known. Most likely it is a form of flocculation, the destabilization of a suspension of colloids by the addition of a chemical agent known as a flocculant [57]. Charged polymers are commonly used as flocculating agents in the treatment of wastewater, to purify proteins, and to control the surface properties of biopharmaceuticals [58-63]. Consistent with our results, several studies have observed that polymer flocs bind large particles and proteins more readily than smaller ones [60-62, 64-66]. The selective incorporation of large particles into polymer flocs appears to be a consequence of the kinetics of polymer adsorption. The rate of polymer adsorption, which is the result of particle-polymer collisions that occur due to Brownian motion or agitation of the suspension, increases with particle size. Since polymer adsorption is effectively irreversible, adsorbed polymers do not later redistribute to particles of smaller sizes. As a result, the number of polymer molecules adsorbed per particle increases with the size of the particles, which causes the larger particles to be preferentially flocculated [67].

These studies suggest that retroviruses are selectively incorporated into complexes of PB and CSC because of their large size. If this is the case, then it is likely that polymer complexation can be used to purify retroviruses from many other types of substances, in addition to free envelope proteins. For example, polymer complexation may prove useful for purifying retroviruses from substances that inhibit transduction that are not envelope proteins, such as TGF-beta or other unidentified inhibitors of transduction such as those secreted by Chinese hamster ovary cells [68, 69]. Polymer complexation may also prove useful for purifying retroviruses from molecules in virus stocks that do not inhibit transduction but nevertheless reduce the chances of success in a gene therapy protocol. For example, cells that produce retroviruses that encode a cytotoxic protein, such as fas ligand [70], may express and secrete into the virus stock the cytotoxic protein or its derivative, which makes it necessary to purify the virus from these substances before it is used to transduce cells. Virus stocks can also contain molecules, such as those found in stocks produced by HT1080 cells, that prevent hematopoietic stem cells from engrafting or induce them to differentiate in an uncontrolled manner [31, 71]. Further experiments are needed in order to determine the extent to which polymer complexation can be used to purify retroviruses from these and other substances that contaminate retrovirus stocks.

We compared the effectiveness of retrovirus purification by complexation with polymers to retrovirus purification by ultracentrifugation and overnight low speed centrifugation. Interestingly, we found that retrovirus purified by complexation with polymers transduced cells 2 to 4-fold more efficiently than retrovirus purified by these other methods. Most likely this is because the polymer-purified retroviruses were still associated with polymer complexes when they were applied to the cells, and therefore were transported more rapidly to the surfaces of the cells (refer to Chapter 3) [35]. It is also possible that ultracentrifugation and low speed centrifugation only partially purify

retroviruses from inhibitors, in which case additional processing steps, such as chromatography or sucrose banding after centrifugation, may be required before using them to transduce cells [22, 25].

In summary, we have shown that retroviruses can be rapidly purified from inhibitors of transduction by complexation with CSC and PB. Purification of retrovirus by polymer complexation has a number of advantages: 1) retroviruses can be purified in larger volumes and much more rapidly than by ultracentrifugation or overnight low speed centrifugation, 2) retroviruses do not need to be pseudotyped with a specific envelope protein in order to be purified, and 3) retroviruses purified by polymer complexation transduce cells more efficiently than retrovirus purified by ultracentrifugation or overnight low speed centrifugation. Purification steps such as these are important for the formulation of retrovirus preparations that are suitable for use in human gene therapy protocols. Purified virus transfers more genes, can be used in smaller quantities to achieve the desired level of gene transfer, and most likely will improve the safety of these procedures by improving the reproducibility and predictability of their outcomes.

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CHAPTER 6

RELATIONSHIP BETWEEN GENE TRANSFER AND NUMBER OF ENVELOPE PROTEINS INCORPORATED PER RETROVIRUS PARTICLE WHEN HIGH DOSES OF PURIFIED VIRUS STOCKS ARE USED TO TRANSDUCE CELLS

6.1 Abstract

The efficiency of retroviral mediated gene transfer is in part determined by the interaction between viral envelope glycoproteins and their cognate cellular receptors. While increasing the concentration of cell receptors usually results in enhancement of transduction, it is not clear how varying the concentration of virus envelope protein (gp70) affects gene transfer. Measurements of titer with diluted virus stocks have shown that increasing the concentration of gp70 increases the efficiency and the rate of transduction. However, titer accounts for transduction of a single cell with a single isolated virus. These conditions do not mimic a typical gene transfer protocol, where undiluted stocks are used. This observation prompted us to analyze how varying the number of envelope proteins per virus affects transduction with undiluted virus stocks. The results from previous research are conflicting and suggest that gene transfer does not directly correlate with the concentration of gp70. In this study, we tested the hypothesis that an excess of virus-associated envelope glycoprotein limits the efficiency of transduction. To test this hypothesis, we constructed a panel of several clonal amphotropic murine leukemia virus packaging cell lines, each of which was derived from the same parental cell line and differed only in the amount of envelope protein they expressed. We purified the stocks produced by these cell lines from free envelope protein and analyzed the effect of virus-associated envelope on transduction. Stocks with low or high number of envelope proteins per virus achieved almost 100%

transduction. However, stocks containing low concentrations of virus-associated envelope achieved the highest efficiencies of transduction, most likely by allowing multiple integrations of the transgene per target cell. High concentrations of virus-associated amphotropic envelope inhibited transduction in a receptor dependent manner. Taken together, our results suggested that while optimum gene transfer is achieved when the number of envelope proteins per virus is low, viruses displaying high concentrations of the envelope can mediate efficient transduction, while limiting the number of gene copies delivered per target cell.

6.2 Introduction

Recombinant retroviruses are commonly used as gene delivery vectors, mainly because they are able to stably integrate their genes into the genome of the target cells [1, 2]. Unfortunately, the efficiency of gene transfer using this vector is often too low to achieve an intended therapeutic effect. In addition, the level of transduction with retroviruses has proven difficult to predict and control. Predictable and reproducible levels of gene transfer are important in order to maximize the benefits of retrovirus transduction while minimizing the likelihood of any adverse side effects. In order to develop therapies that are effective, safe and reproducible, we require a better understanding of major rate-limiting steps of transduction.

One of the earliest and critical steps of transduction is the specific interaction between the envelope glycoproteins of the virus and their cognate receptors on the surface of the target cells. This interaction mediates fusion and entry of the viral core into the cytoplasm of the cell. Also, it is a major determinant of the tropism of the virus (the range of cells that a virus can or cannot transduce). In order to target specific cells in gene therapy protocols, the wild type envelope of a virus has been frequently replaced with one from another virus to form a pseudotyped virus (a virus composed of proteins

from more than one virus) [3, 4]. Pseudotyping has also been exploited as a means to enhance the efficiency of gene transfer [5, 6], by targeting receptors that are expressed at high concentrations. In fact, varying the extent of receptor-envelope interactions can affect the overall efficiency of transduction. A better understanding of how gene transfer responds to changes in the absolute and relative concentrations of cellular receptors and viral envelope proteins can therefore be used to improve the reproducibility and predictability of gene transfer.

High expression of receptors for a particular envelope appears to increase the likelihood of gene transfer [7-13]. In some cases, transduction has been enhanced by inducing overexpression of receptors through manipulation of the cell culture medium. For example, the amphotropic receptor (Pit-2) and the gibbon ape leukemia virus receptor (Pit-1) are upregulated by incubation of the cells in phosphate depleted medium or in the presence of phorbol 12-myristate 13-acetate (PMA) [10, 14-16]. In hematopoietic stem cells, these receptors can be upregulated by incubation of the cells with cytokines [12, 13, 17]. Alternatively, a receptor can be overexpressed by transfecting cells with the gene encoding for it [13, 16]. Slight upregulation of receptors that are normally expressed at low levels has been shown to translate in major increases in gene transfer [13].

The efficiency of envelope-receptor interactions not only depends on the number of cell receptors, but can also be altered by varying the number of envelope glycoproteins per virus particle [18]. The level of incorporation of envelope proteins on the surface of a virus depends on the level of expression of these proteins by packaging cells [18, 19] and on the recognition of the envelope by the viral core during assembly of the virion [20-22]. During transduction, envelopes from a single virus form oligomers and act cooperatively to achieve efficient fusion [22-24]. Although the exact mechanism is not clear, it has been proposed that the rate of fusion depends on how tightly the virus

binds to the cell, which is turn determined by the number of interactions between envelope proteins from a virus and cell receptors [18, 25]. As a result, higher concentrations of virus-associated envelope proteins have been shown to correlate with higher rates of transduction. In agreement with this idea, it has been possible to enhance gene transfer by increasing the fluidity of the cellular plasma membrane [25, 26]. A more fluid membrane appears to favor rapid mobilization of receptors to the site of virus binding, thereby providing multiple binding sites for multiple envelope proteins of a virus.

Although the rate of a transduction event increases with increasing concentrations of envelope proteins, low concentrations, above a certain threshold, have proven sufficient to achieve optimal titers [18]. Titers are measured using very diluted virus stocks [18]. When using undiluted or less diluted virus stocks, the results from various studies are inconsistent. In some cases, increasing the concentration of viral envelope has been shown to enhance the efficiency of gene transfer [20, 21]. This enhancement using the simian immunodeficiency virus envelope was only possible when receptors were expressed at high levels. At low levels of receptors, increasing the concentration of viral envelope did not have any effect on transduction [21]. In contrast, Slingsby et al. showed an inverse correlation between the concentration of amphotropic envelope proteins and the efficiency of transduction [19]. They proposed that there is an optimum level of expression of envelope proteins that maximizes transduction. This optimum level can be in part dictated by the concentration of free envelope. Free envelope can be shed from packaging cells and from viruses, and act as a competitive inhibitor of transduction by binding to cellular receptors [7-9, 24, 27, 28]. In addition, envelope proteins can associate with defective virus particles lacking the viral vector, and also compete with functional viruses for cell receptors [19].

Overall, the results from titer and from transduction using undiluted stocks are somewhat contradictory and difficult to compare. Titer has often been used to predict the

efficiency of transduction with undiluted virus stocks. However, to measure titer, virus stocks are diluted several thousand fold in fresh medium, such that each cell is likely to encounter at most one virus particle throughout the transduction experiment and the composition of the medium is optimum for the intended target cell. On the contrary, undiluted virus stocks are typically used in human gene therapy protocols, such that an individual cell may encounter many viruses within a short period of time. Transduction with undiluted virus stocks can be influenced by the composition of the medium, which can vary from harvest to harvest of the same virus. For example, some stocks contain higher concentrations of free envelope inhibitor than others. These differences can be eliminated by purifying viruses from the conditioned medium and suspending them in fresh cell culture medium (refer to Chapter 5). However, there is a possibility that when multiple viruses bind simultaneously to a single cell, the susceptibility of the cell to transduction is regulated by factors inherent to the viruses themselves, for example by the number of envelope proteins per virus. Given that in a typical gene transfer protocol virus stocks are not diluted but can be purified and concentrated, there is significant motivation to investigate how varying characteristics of the virus particles affect transduction. In this context, we hypothesized that increasing the concentration of virus-associated envelope proteins above a certain threshold hinders transduction.

6.3 Materials and methods

Chemicals and antibodies. Chondroitin sulfate C (CSC) (shark cartilage), gluteraldehyde, 1,5-dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene, PB) and o-Nitrophenyl β-D-galactopyranose (ONPG) were from Sigma Chemical Co. (St. Louis, MO). o-Phenylenediamine Dihydrochloride (OPD) was from Pierce (Rockford, IL). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was from Denville Scientific,

Inc. (Metuchen, NJ). Mouse anti-p30 and mouse anti-gp70 antibodies were purified from the supernatant of the CRL-1219 (ATCC, Rockville, MD) and the 83A25 [29] hybridoma cell lines respectively, following standard procedures [30]. The goat polyclonal anti-p30 antibody (78S221) and the goat polyclonal anti-gp70 (79S834) were from Quality Biotech (Camden, NJ). The horseradish peroxidase conjugated rabbit anti-goat immunoglobulin G polyclonal antibody was from Zymed Laboratories (South San Francisco, CA).

Cell culture. NIH 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Labs Inc., Logan, UT) with 10% bovine calf serum (Hyclone Labs Inc., Logan, UT), 100 U/mL of penicillin and 100 μg/mL of streptomycin (Hyclone Labs Inc.) (DMEM/BCS). TE671 (human medulloblastoma epithelial cells), TELCeB6 (TE671 cells expressing Mo-MLVgagpol and the retroviral vector MFGnlsLacZ [31]), and HeLa cells (human adenocarcinoma cells), obtained from the ATCC, were cultured in DMEM, 10% fetal bovine serum (Hyclone Labs Inc.), 100 U/mL of penicillin and 100 μg/mL of streptomycin (DMEM/FBS). GP2-293 packaging cells (HEK 293 expressing Mo-MLVgagpol, BD Biosciences, Franklin Lakes, NJ) were cultured in DMEM/FBS, in collagen coated Biocoat culture plates (BD Biosciences).

Generation of stable amphotropic retrovirus packaging cell lines. An amphotropic packaging cell line (TELCeB6-A) was generated by stable transfection of TELCeB6 cells. Five micrograms of the plasmid FB4070ASALF, an expression plasmid that encodes for the amphotropic envelope glycoprotein (a kind gift of Stephen Russell), was dissolved in 400 μL of 0.25M $CaCl_2$, mixed with 400 μL of 2X HEPES buffered saline (274 mM NaCl, 42 mM Hepes acid, 10 mM KCl, 1.4 mM Na_2HPO_4 and 12 mM dextrose), incubated at room temperature for 20 minutes, then added to a 50% confluent T75 flask of TELCeB6 cells. Twelve hours after transfection the cells were washed with

PBS, and then the medium replaced with fresh DMEM/FBS. Two days later the cells were trypsinized, pelleted, and resuspended. Two hundred microliters of the resuspended cells were diluted in 10 mL of selective medium (DMEM, 10% FBS, 50 μg/mL of phleomycin, and 7 μg/mL of blasticidin), plated in a T75 flask, and the selective medium was replaced every 2-3 days. Fourteen days later pooled clones were collected and plated at clonal density. Ten days later, individual clones were selected, expanded and frozen (-80°C) for later use.

Production of retroviruses. To generate retrovirus stocks from stable packaging cells, virus-producing cells were grown to confluence in T175 tissue culture flasks, then incubated for 24 h with 35 mL of cell culture medium. The virus-laden tissue culture medium was harvested, filter sterilized (0.45-μm), then frozen (-80°C) for later use. We produced amphotropic and ecotropic retrovirus vectors encoding for GFP by transient transfection of GP2-293 cells that were plated the day before in 10-cm tissue culture dishes ($6x10^6$ cells/dish), and cultured in 10 mL of DMEM that contained no antibiotics. The next day (between 18 to 30 h later), cells were transfected with FB4070ASALF (for amphotropic virus) or FBMO-SALF (for ecotropic virus) and the MSCV-GFP vector (kind gift of Catherine Verfaillie). Eight micrograms of each plasmid were diluted into a total of 1.5 mL of DMEM, then mixed with a solution of 1.5 mL of DMEM and 40 μL of Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA), then added to the cells. The medium was replaced 6 hours later and 18 hours later with 10 mL of DMEM/FBS. Twenty four hours later the virus-laden cell culture supernatant was harvested, filter sterilized (0.45-μm) and frozen (-80°C) for later use.

ELISA for p30 and gp70. We used an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of virus capsid protein (p30) and the viral envelope glycoprotein (gp70). ELISA plates (Nunc immuno Maxisorp 96-well plates,

Nalge Nunc International, Rochester, NY) were coated overnight at 4°C with 10 μg/mL of mouse anti-p30 antibody or mouse anti-gp70 antibody (100 μL/well) in PBS. The next day, the antibody solution was removed and blocking buffer (PBS, 0.05% Tween-20, 5% non-fat milk) added (200 μL/well) for 2 h at 37°C to block non-specific binding sites. Samples were brought to 0.5% Triton-X to expose the p30 antigen, then added to the ELISA plate (100 μL/well) and incubated for 1 h at 37°C. Bound p30 and gp70 were sandwiched by the addition of the goat polyclonal anti-p30 antibody diluted 1:1000 in blocking buffer or the goat polyclonal anti-gp70 diluted 1:300 in blocking buffer, and incubated for 1 h at 37°C. The horseradish peroxidase conjugated polyclonal rabbit anti-goat immunoglobulin G was diluted 1:5000 in blocking buffer then added to the ELISA plate (100 μL/well) for 1 hour at 37°C to enable detection and quantitation of the sandwiched p30 antigen. The plates were developed using hydrogen peroxide (H_2O_2) and OPD (100 μg/well) from a solution of 10 mg of OPD and 10 μl H_2O_2 in 25 mL of substrate buffer (24 mM citric acid-monohydrate, 51 mM Na_2HPO_4-$7H_20$, pH 5.0). 8N sulfuric acid (50 μL/well) was used to stop the reaction and the optical density at 490 nm (OD_{490}) measured using an absorbance plate reader and the non-specific background at 650nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the average of at least triplicate wells.

Diluted titer assay. Ten-fold serial dilutions of *lacZ* virus stock were made in DMEM/BCS and Polybrene (8 μg/mL). A 1-mL amount per well was used to transduce 3T3 cells that had been seeded (7×10^4 per well) the previous day in a 12-well plate. Two days after the start of the transduction, the cells were fixed and stained for β-galactosidase activity with X-Gal [32]. Colonies of *lacZ*+ cells (typically in clusters of 2, 4, or 8 blue cells) were counted with the aid of a dissecting microscope. At appropriate dilutions of the virus stock, the clusters of blue cells were sufficiently spread over the

dish such that each cluster arose from a single transduction event. From triplicate wells, the number of *lacZ*+ CFU per milliliter was determined.

Transduction assay. Five thousand NIH 3T3 murine fibroblasts in 100 μL of medium were plated per well in a 96-well flat-bottomed tissue culture dish with a low-evaporation lid (Costar Corp., Cambridge, MA). The next day (19 to 26 h later), the medium was removed and 100 μL of the virus-containing solutions added to each well. Two days after transduction, the medium was removed and the cells washed once with 100 μL of phosphate-buffered saline (PBS) containing 1mM $MgCl_2$. After removal of the wash solution, 50 μL of lysis buffer (PBS with 1mM $MgCl_2$ and 0.5% Igepal) were added to each well, and the plate incubated at 37°C. After 30 min, 50 μl of lysis buffer with 6 mM ONPG warmed to 37°C were added to each well, and the plate incubated at 37°C for 5 to 60 min until a visible yellow color was obtained. The reactions were halted by the addition of 20 μL per well of stop buffer (1M Na_2CO_3). The optical density at 420 nm (OD_{420}) was measured using an absorbance plate reader (Molecular Devices, Menlo Park, CA) and the non-specific background at 650 nm subtracted. Values for replicate wells without virus were subtracted as background. Values for each point are the averages of at least triplicate wells.

Purification of virus stocks.

Purification by complexation with polymers. Stocks were purified as previously described [33]. Briefly, stocks were brought to equal weight concentrations (80 μg/mL) of PB and CSC by adding, in immediate succession, appropriate volumes of aqueous stock solutions (20 mg/mL) of the polymers and vortexing the mixture. The mixture was incubated for 20 min at 37°C, then centrifuged at 10000 rpm for 5 min. The supernatant was aspirated and the pellet was resuspended to the original volume in fresh cell culture medium.

Purification by low speed centrifugation. Virus stocks were centrifuged for 16 hrs at 6000g, 4°C. The supernatant was decanted and the pellet resuspended to the original volume in fresh cell culture medium.

Percent transduced assay. Cells were transduced in 96-well dishes as described above, grown to confluence, and trypsinized. Twenty to one hundred microliters of the trypsinized cells were diluted into 1 mL of medium. Ten microliters of this dilution was plated into a 10-cm dish. After 10 days, when macroscopic colonies had grown, the plates were stained for β-galactosidase as described in the titer assay. The colonies were then counterstained with 1% rhodamine B, rinsed with water and air dried. Values for the percentage of lacZ+ colonies at each point (blue colonies/total colonies x 100) are the averages of triplicate wells of the 96-well dish.

Fluorescence microscopy. NIH 3T3 cells were transduced as previously described with viruses carrying the GFP gene. Two days later, fluorescent images of the cells were taken using an inverted fluorescence microscope (IX-50, Olympus America, Inc., Melville, NY). The percentage of GFP+ cells was measured using the image analysis software MetaMorph Offline (Version 6.1 r0).

Rate of binding of viruses to cells. NIH-3T3 cells were plated in 96-well dishes (5000 cells per well). The next day the medium was removed and replaced with 100 μL per well of virus purified by pelleting with polymers. At various times, the supernatant was collected. The concentration of p30 remaining in the supernatant was measured by ELISA. The rate of disappearance of p30 from solution has been shown to correlate with the rate of binding of p30 to cells (refer to Chapter 3)[34].

6.4 Results

To study how varying the concentration of viral envelope glycoprotein (gp70) affects the efficiency of retroviral-mediated gene transfer, we constructed a panel of several clonal amphotropic murine leukemia virus packaging cell lines, each of which were derived from the same parental cell line (TELCeB6 cells). These packaging cell lines (TELCeB6-A) differed only in the amount of envelope protein they expressed. We measured the concentrations of virus capsid protein (p30) and viral envelope protein (gp70) in virus stocks produced by each clonal packaging cell line and chose stocks with different concentrations of gp70 per p30 for further analysis.

To determine the extent to which varying the concentration of gp70 affected the bioactivity of individual virus particles, we measured the titer of the stocks by transducing NIH 3T3 fibroblasts with stocks that had been diluted several thousand fold. We found that titer, and therefore the bioactivity of viruses, increased with increasing concentrations of gp70 and eventually reached a plateau (Figure 6.1).

The results from titer represent the efficiency of transduction with a very low concentration of viruses and in a medium that is optimum for the target cells. In order to assess how varying the concentration of gp70 affects transduction in a typical gene transfer setting, we transduced NIH 3T3 cells with undiluted stocks produced from TELCeB6-A clones. Transduction was maximal at concentrations of gp70 corresponding to titers of 1.4×10^7 CFU/mL, and decreased to very low levels at concentrations of gp70 that achieved titers of 3.4×10^7 CFU/mL (Figure 6.2). These results showed that indicated that an excess of gp70 in the stocks hinders gene transfer.

The concentration of gp70 in virus stocks corresponds to the sum of virus-associated gp70 and free gp70. The inhibitory effect of free gp70 is negligible when measuring titer, where the stock is diluted several thousand-fold prior to transduction. We therefore examined the possibility that, when using undiluted stocks, the decrease in

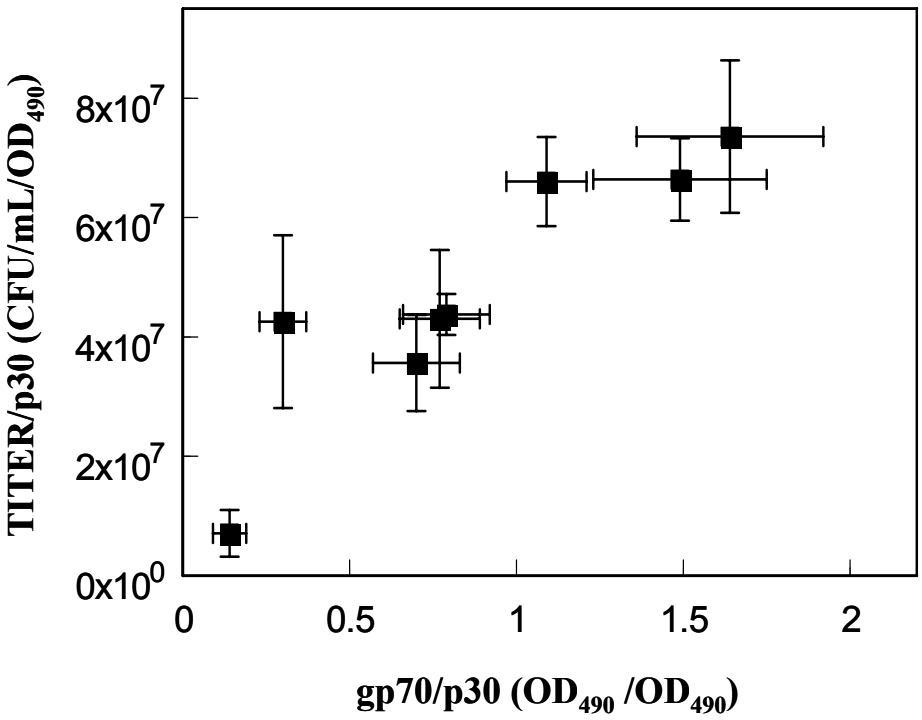

Figure 6.1 Titer increases with increasing concentrations of gp70 and reaches a plateau. Virus stocks produced from various clones from TELCeB6-A were diluted several thousand fold in fresh medium, then brought to 8 μg/mL of PB and used to transduced NIH 3T3 fibroblasts. The titer was normalized with the concentration of p30 in the stocks. Each point shows the mean ± standard deviation of three replicates.

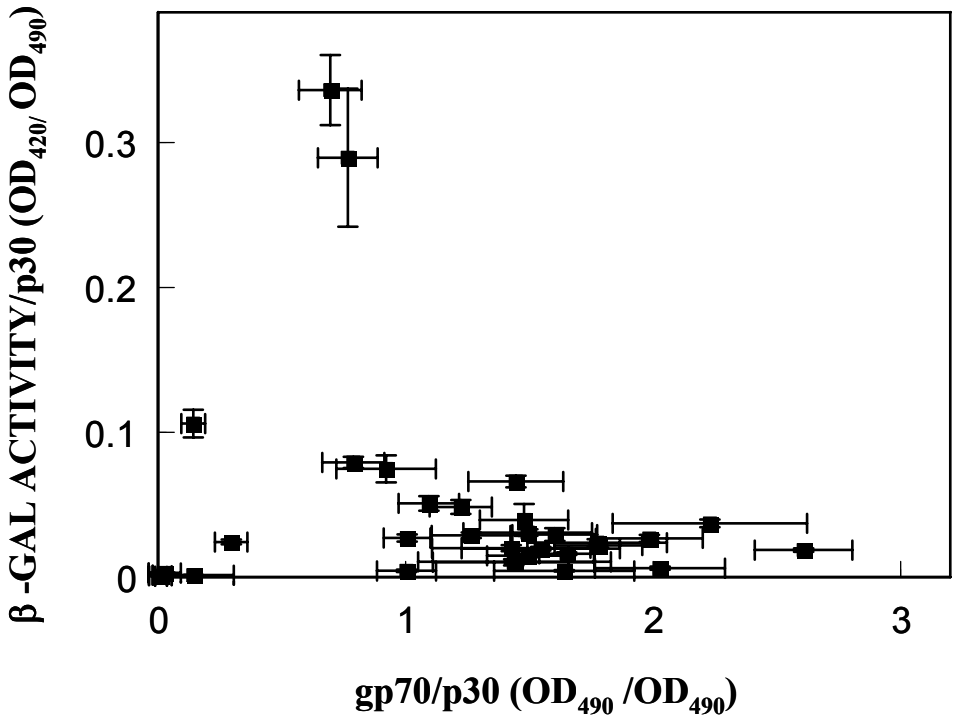

Figure 6.2 Transduction with undiluted virus stocks is maximal at low concentrations of gp70. Undiluted virus stocks were brought to 8 μg/mL of PB, then used to transduce NIH 3T3 fibroblasts. The efficiency of gene transfer was normalized with the concentration of p30 in the stocks. Each point shows the mean ± standard deviation of three replicates.

gene transfer at high concentrations of gp70 was due to inhibition by free gp70. We purified stocks from free gp70 by complexation of viruses with charged polymers, as previously described (refer to Chapter 5). Briefly, we induced aggregation of viruses into polyelectrolyte complexes by adding the cationic polymer Polybrene and the anionic polymer chondroitin sulfate C to virus stocks. Then, we pelleted the complexes, decanted the supernatant and resupended the pellet to its original volume in fresh cell culture medium. We transduced cells with purified virus stocks and found that the efficiency of gene transfer increased significantly with all the stocks (compare Figures 6.2 and 6.3). However, gene transfer was still maximal at low concentrations of gp70 and decreased at high concentrations of gp70 (Figure 6.3). These results suggested that an excess envelope proteins associated with virus particles hinders gene transfer when cells are exposed to undiluted virus stocks.

To investigate the mechanism by which increasing the number of envelope proteins per virus particle reduced transduction, we selected purified virus stocks from four TELCeB6-A clones that expressed different concentrations of gp70, but similar concentrations of p30: clones 1-18, 2-17, 2-18 and 2-44 (Table 6.1). In Figure 6.3, the stock from clone 1-18 is at the left of the peak, the stock from clone 2-17 is at the peak and the stocks from clones 2-18 and 2-45 are at the right of the peak.

Given that increasing the concentration of gp70 enhanced transduction with very diluted stocks (Figure 6.1) but decreased transduction with undiluted stocks (Figure 6.3), we decided to examine in more detail the relationship between the level of gene transfer and the concentration of virus particles from each of the four selected stocks. We brought a range of volumes of each virus stock (0 to 100 μL) to 100 μL with fresh medium and then used the solutions to transduce NIH 3T3 cells. When using stocks with a low number of gp70 per virus (produced by clone 1-18), gene transfer increased linearly with increasing concentrations of viruses (Figure 6.4). However, when using

Table 6.1 Selected TELCeB6-A clones

Clone	Relative number of gp70 per virus [a,c]	Relative concentration of p30 [b,c]	Titer (CFU/mL)
1-18	1.00 ± 0.29	1.00 ± 0.07	$0.29 \times 10^7 \pm 0.02 \times 10^7$
2-17	3.29 ± 0.87	1.00 ± 0.08	$1.47 \times 10^7 \pm 0.25 \times 10^7$
2-18	16.77 ± 3.66	1.1 ± 0.10	$3.08 \times 10^7 \pm 0.29 \times 10^7$
2-44	22.32 ± 4.90	1.04 ± 0.07	$3.38 \times 10^7 \pm 0.20 \times 10^7$

a: Virus produced by TELCeB6-A clones cells was purified by complexation with polymers. Purified virus was analyzed for its concentration of p30 and gp70 by ELISA. The number of gp70 per virus was determined as the ratio of gp70 to p30. Values are the means ± SD for three replicates.
b: Virus produced by TELCeB6-A clones cells was purified by complexation with polymers. Purified virus was analyzed for its concentration of p30 Values are the means ± SD for three replicates.
c: Relative to values in clone 1-18.

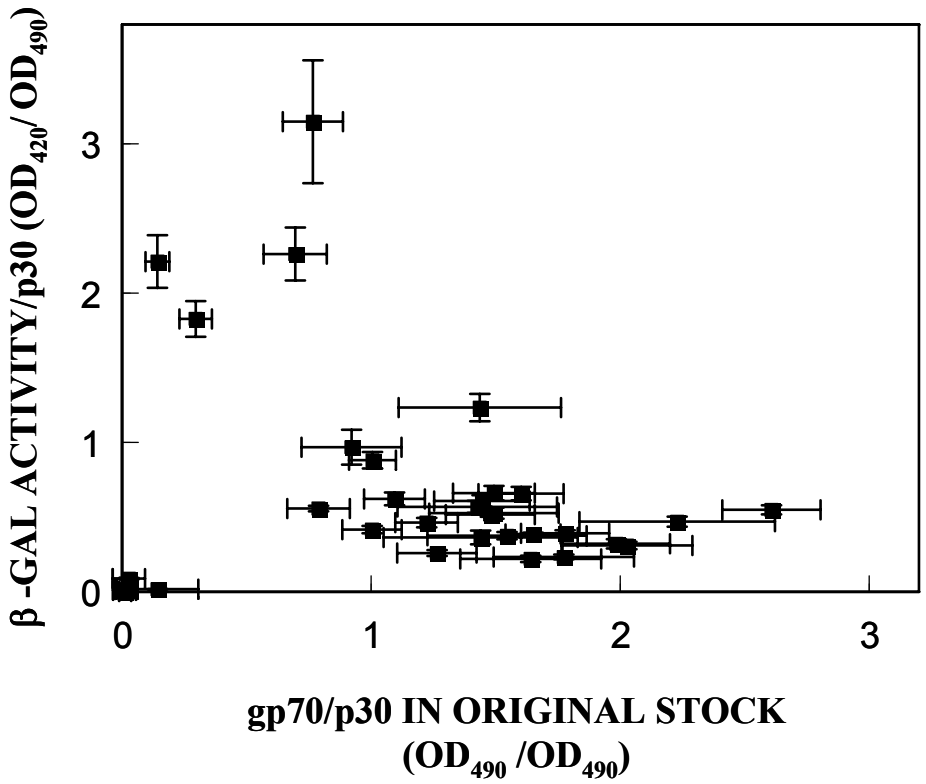

Figure 6.3 Transduction with purified virus stocks is maximal at low concentrations of gp70. Virus stocks purified by complexation with polymers were brought to 8 μg/mL of PB, then used to transduce NIH 3T3 fibroblasts. The efficiency of gene transfer was normalized with the concentration of p30 in the stocks. Each point shows the mean ± standard deviation of three replicates.

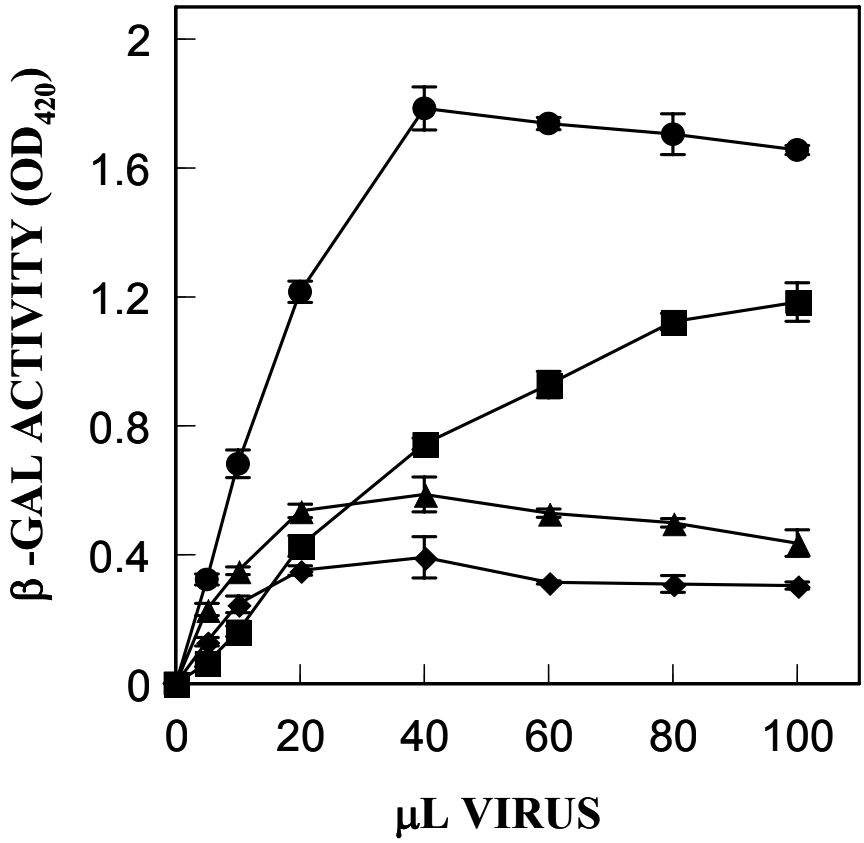

Figure 6.4 Low concentrations of viruses displaying high numbers of envelope proteins are required to achieve maximal gene transfer. Virus stocks produced by TELCeB6-A clones 1-18 (■), 2-17 (●), 2-18 (▲) and 2-44 (◆) were purified by complexation with polymers, then diluted in fresh medium and used to transduce NIH 3T3 cells. The efficiency of gene transfer was quantified. Each point shows the mean ± standard deviation of three replicates.

stocks with higher numbers of gp70 per virus (produced by clones 2-17, 2-18 and 2-44), the relationship between virus dose and gene transfer was linear only when low doses of virus were used (20 to 40 μL per well). When higher doses of virus were used, transduction reached a plateau and remained virtually constant (Figure 6.4). Moreover, this plateau developed earlier as the number of envelope proteins per virus increased (Figure 6.4).

This limit in gene transfer at high concentrations of virus-associated gp70 could be due to a decrease in the number of cells susceptible to transduction or to a decrease in the susceptibility of individual cells to multiple transduction events. To assess these possibilities, we conducted a parallel experiment to that from Figure 6.4, where we quantified the fraction of cells transduced as a function of virus concentration. Transduction with only 20 to 40 μL of the virus stocks was sufficient to achieve almost 100% transduction, regardless of the number of envelope proteins per virus (Figure 6.5). Remarkably, when using the stock from clone 1-18, the level of expression of the transgene continued to increase in spite of having reached 100% transduction (compare Figures 6.4 and 6.5). This indicated that when the number of envelope proteins per virus was low, each cell integrated more copies of the transgene than when the number of envelope proteins per virus was high.

To assess if these findings were specific to NIH 3T3 target cells, we examined if high concentrations of virus-associated gp70 would limit transduction to other cell lines. We brought a range of volumes (0 to 100 μL) of virus stock produced by clones 1-18, 2-17 and 2-44 to 100 μL with fresh medium and then used these solutions to transduce human rhabdomyosarcoma (TE671) and human adenocarcinoma (Hela) cells. As with NIH 3T3, the efficiency of transduction increased with increasing concentrations of virus and reached a plateau (Figures 6.6 and 6.7). However, the plateaus were reached

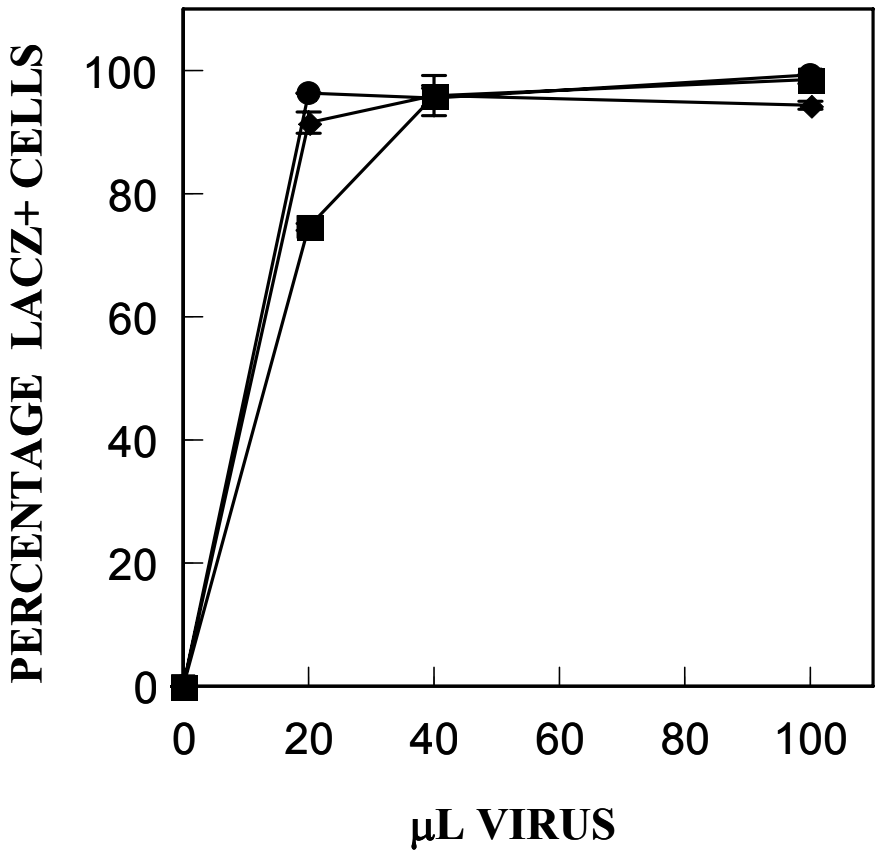

Figure 6.5 Low concentrations of viruses displaying high numbers of envelope proteins are required to transduce 100% of the cells. Virus stocks produced by TELCeB6-A clones 1-18 (■), 2-17 (●), 2-18 (▲) and 2-44 (◆) were purified by complexation with polymers, then diluted in fresh medium and used to transduce NIH 3T3 cells. The percentage of lacZ+ cells was quantified. Each point shows the mean ± standard deviation of three replicates.

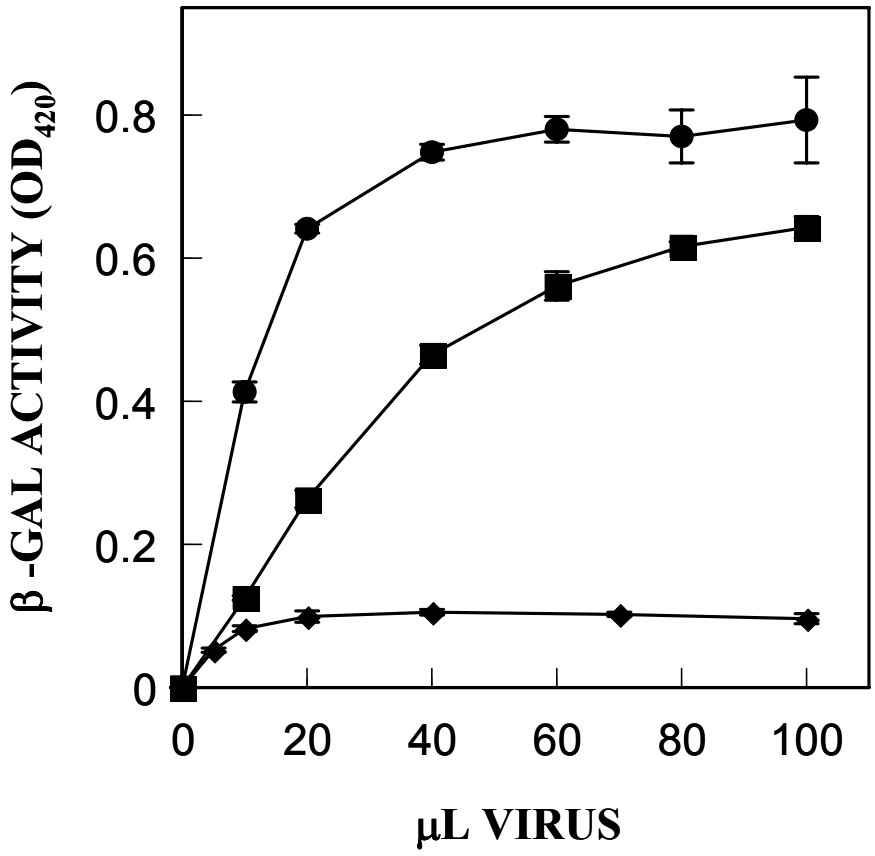

Figure 6.6 High concentrations of virus-associated gp70 limit gene transfer to TE671 cells. Virus produced by TELCeB6-A clones 1-18 (■), 2-17 (●),and 2-44 (◆) were purified by complexation with polymers, then diluted in fresh medium and used to transduce TE671 cells. Each point shows the mean ± standard deviation of three replicates.

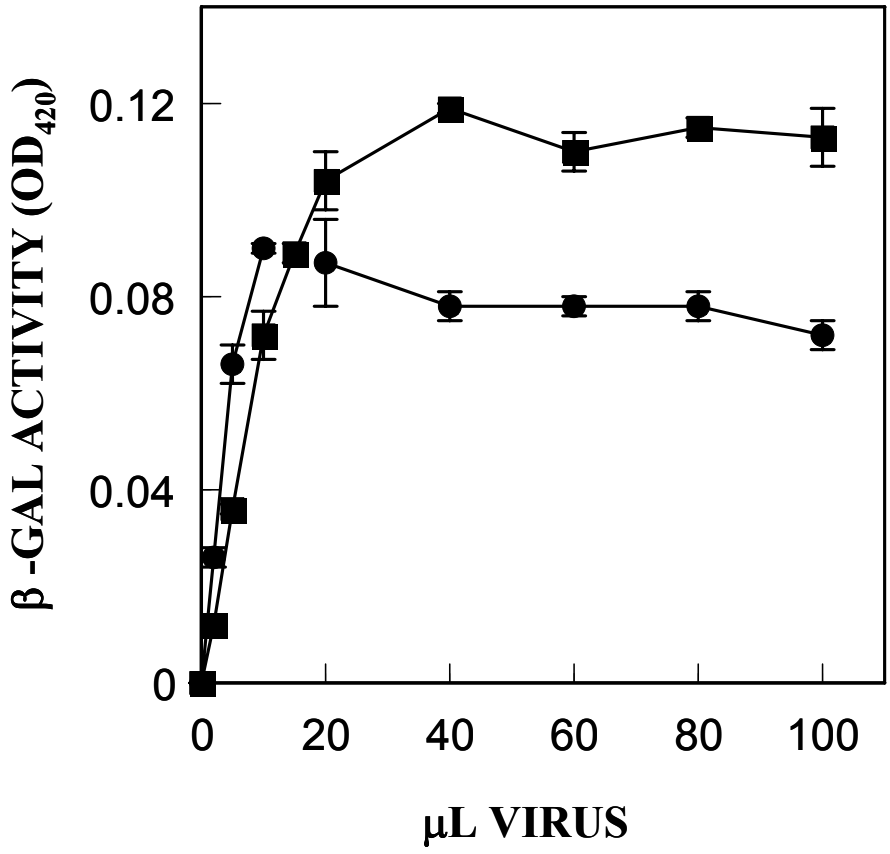

Figure 6.7 High concentrations of virus-associated gp70 limit gene transfer to Hela cells. Virus produced by TELCeB6-A clones 1-18 (■), 2-17 (●) were purified by complexation with polymers, then diluted in fresh medium and used to transduce Hela cells. Each point shows the mean ± standard deviation of three replicates.

earlier than the plateaus observed with NIH 3T3 cells (compare Figure 6.4 and Figures 6.6-6.7). In the case of Hela cells, transduction was the highest when the number of envelope proteins per virus was the lowest (clone 1-18). In the case of TE671 cells, gene transfer was the highest when the number of envelope proteins per virus was the second lowest (clone 2-17). These results suggested that there is a concentration of virus-associated gp70 that maximizes transduction and that this level depends on the specific target cell line.

Based on these findings, we hypothesized that a high number of envelope proteins per virus was saturating cell receptors and therefore limiting the number of transduction events per target cell. To test this hypothesis, we transduced NIH 3T3 cells with a mixture of 20 μL of amphotropic GFP virus and 80 μL of amphotropic LacZ virus produced by either clone 1-18, clone 2-17 or clone 2-44. As controls, we transduced cells with a mixture of 20 μL of amphotropic GFP virus and 80 μL of fresh medium or with a mixture of 20 μL of amphotropic GFP virus and 80 μL of non-enveloped LacZ viruses. We observed the cells that were transduced with the GFP virus by immunofluorescence and quantified the percentage of GFP-positive cells using image analysis software. We found that as the number of amphotropic envelope proteins per lacZ virus increased, the efficiency of transduction with amphotropic GFP-virus decreased (Figures 6.8 and 6.10). To examine if high concentrations of virus-associated amphotropic envelope could also inhibit gene transfer with viruses displaying a different envelope, we repeated the previous experiment but used ecotropic GFP virus instead of amphotropic GFP virus. We found that the efficiency of transduction with ecotropic GFP did not vary as a function of the number of amphotropic envelope proteins per lacZ virus ($P < 0.5$) (Figures 6.9 and 6.10). These results suggested that high concentrations of envelope proteins that are associated with a virus particle block or otherwise prevent cell

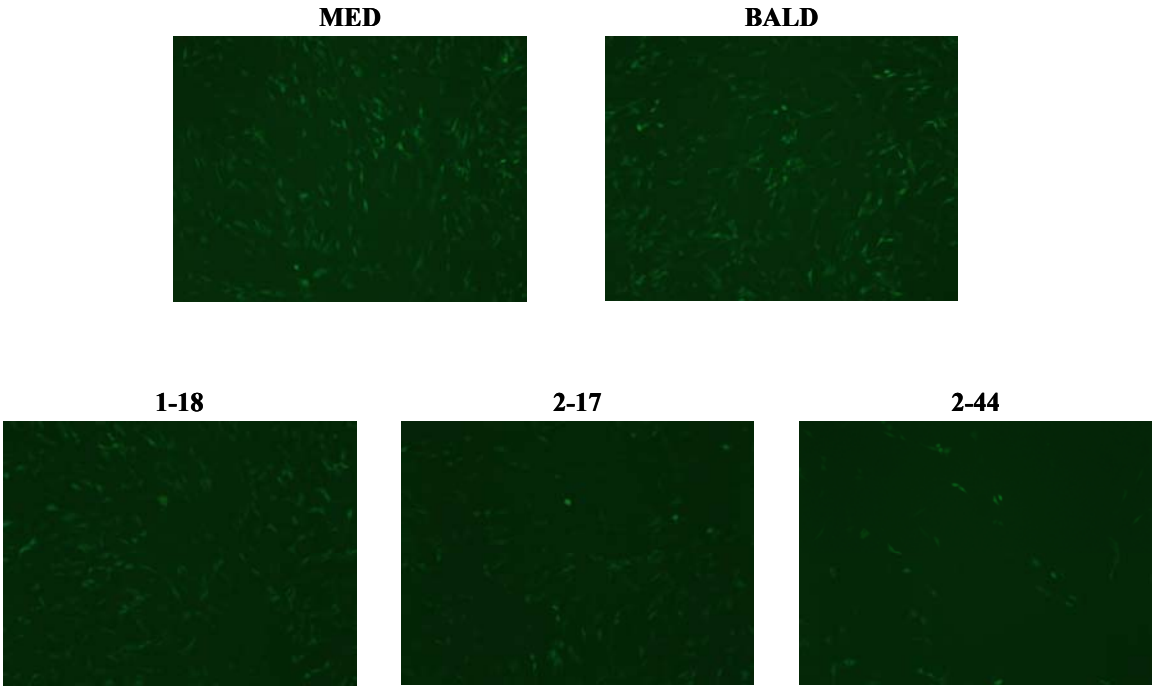

Figure 6.8 High concentrations of virus-associated amphotropic envelope block transduction with amphotropic viruses. NIH 3T3 were transduced with 20 μL of a stock of amphotropic GFP-viruses and 80 μL of a stock of amphotropic LacZ-virus produced by TELCeB6-A clones 1-18, 2-17 or 2-44. As controls, cells were transduced with 20 μL of a stock of amphotropic GFP-viruses and 80 μL of fresh medium (MED), or with 20 μL of a stock of amphotropic GFP-viruses and 80 μL of non-enveloped viruses produced by TELCeB6 packaging cells (BALD). Fluorescent images of GFP+ cells were taken. Note: all the stocks used were purified by complexation with polymers.

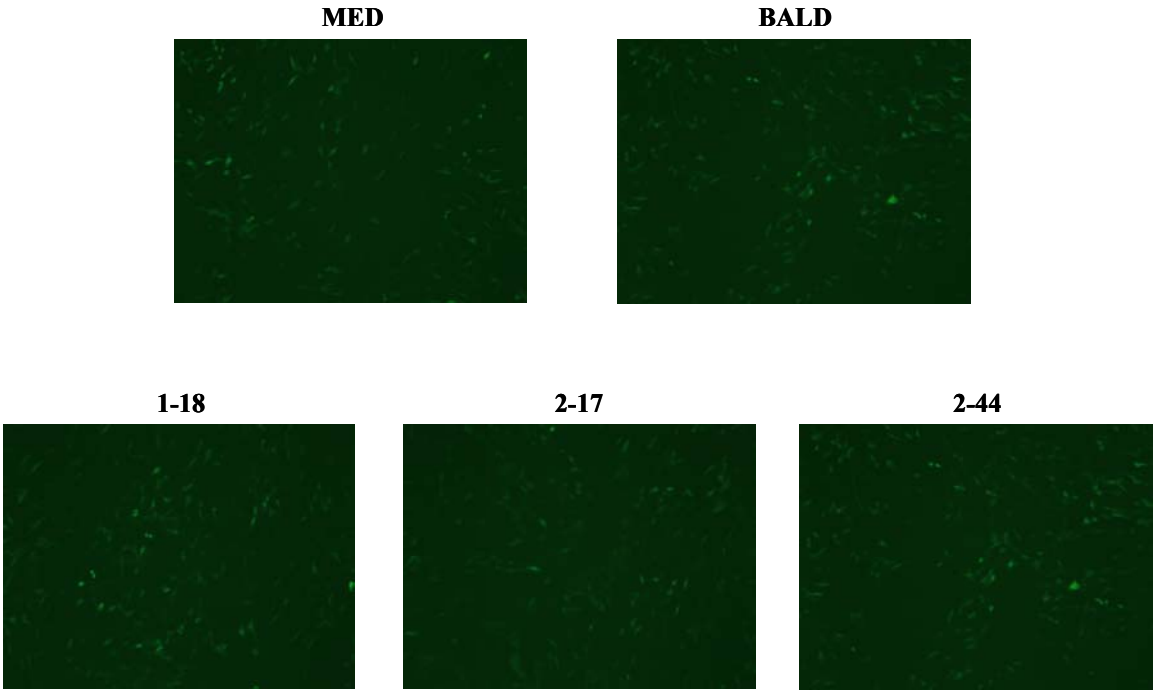

Figure 6.9 High concentrations of virus-associated amphotropic envelope do not block transduction with ecotropic viruses. NIH 3T3 were transduced with 20 μL of a stock of ecotropic GFP-viruses and 80 μL of a stock of amphotropic LacZ-virus produced by TELCeB6-A clones 1-18, 2-17 or 2-44. As controls, cells were transduced with 20 μL of a stock of ecotropic GFP-viruses and 80 μL of fresh medium (MED), or with 20 μL of a stock of ecotropic GFP-viruses and 80 μL of non-enveloped viruses produced by TELCeB6 packaging cells (BALD). Fluorescent images of GFP+ cells were taken. Note: all the stocks used were purified by complexation with polymers.

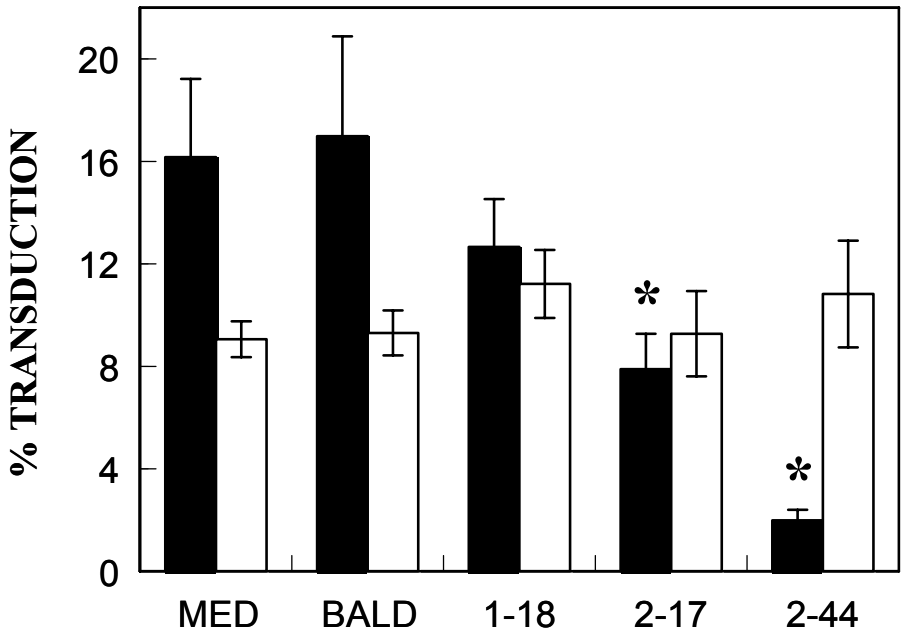

Figure 6.10 High concentrations of virus-associated amphotropic envelope block transduction with amphotropic viruses, but not with ecotropic viruses. NIH 3T3 were transduced with 20 μL of a stock of amphotropic GFP-viruses and 80 μL of a stock of amphotropic LacZ-virus produced by TELCeB6-A clones 1-18, 2-17 or 2-44. As controls, cells were transduced with 20 μL of a stock of amphotropic GFP-viruses and 80 μL of fresh medium (MED), or with 20 μL of a stock of amphotropic GFP-viruses and 80 μL of non-enveloped viruses produced by TELCeB6 packaging cells (BALD). The same experiment was conducted using ecotropic GFP-virus instead of amphotropic GFP-virus. The percentage of fluorescence in wells transduced with amphotropic GFP virus (black bars) or with ecotropic GFP virus (white bars) was quantified by image analysis software. All the stocks used were purified by complexation with polymers. Each point shows the mean ± standard deviation of three replicates. Statistically significant differences ($p \leq 0.05$) from MED are denoted with an asterisk.

surface receptors from interacting with subsequent virus particles that are pseudotyped with the same envelope protein.

In all our previous experiments, we purified virus stocks from free gp70 by inducing aggregation of the viruses into high molecular weight polyelectrolyte complexes. We have shown that viruses within these complexes sediment and are delivered to the cells more rapidly than by simple diffusion, while free retroviruses in conventional transductions are transported to cells by diffusion, a slow process compared to the rate that viruses lose their bioactivity [35]. Increasing the flux of viruses to cells by complexation with polymers has been shown to result in substantial enhancement of transduction (refer to Chapter 3) [34]. However, it was not clear if increasing the rate of virus transport assisted transduction when the number of envelope proteins per virus was high. Since it appeared that only a small fraction of the viruses that reached the cells were able to complete transduction in our system, we hypothesized that at low concentrations of virus-associated gp70, increasing the rate of binding of viruses to cells would not enhance transduction.

To test this hypothesis, we purified virus stocks produced from clones 1-18, 2-17, 2-18 and 2-44 by low speed centrifugation. Viruses purified by this method are not complexed with any agent and their transport is subject to diffusion limitations. To compare the kinetics of binding of viruses purified by complexation with polymers with that of viruses purified by low speed centrifugation, we incubated the purified stocks over NIH 3T3 cells. At various times we measured the concentration of viral capsid protein (p30) that remained in solution. We found that within a half-life of a virus (8 hours), 17% of the virus purified by low speed centrifugation and 74% of the viruses purified by complexation with polymers had disappeared from solution and had adsorbed to the surface of the cells (Figure 6.11). Then, we compared the efficiencies of transduction using viruses purified using these two methods. When the number of envelope proteins

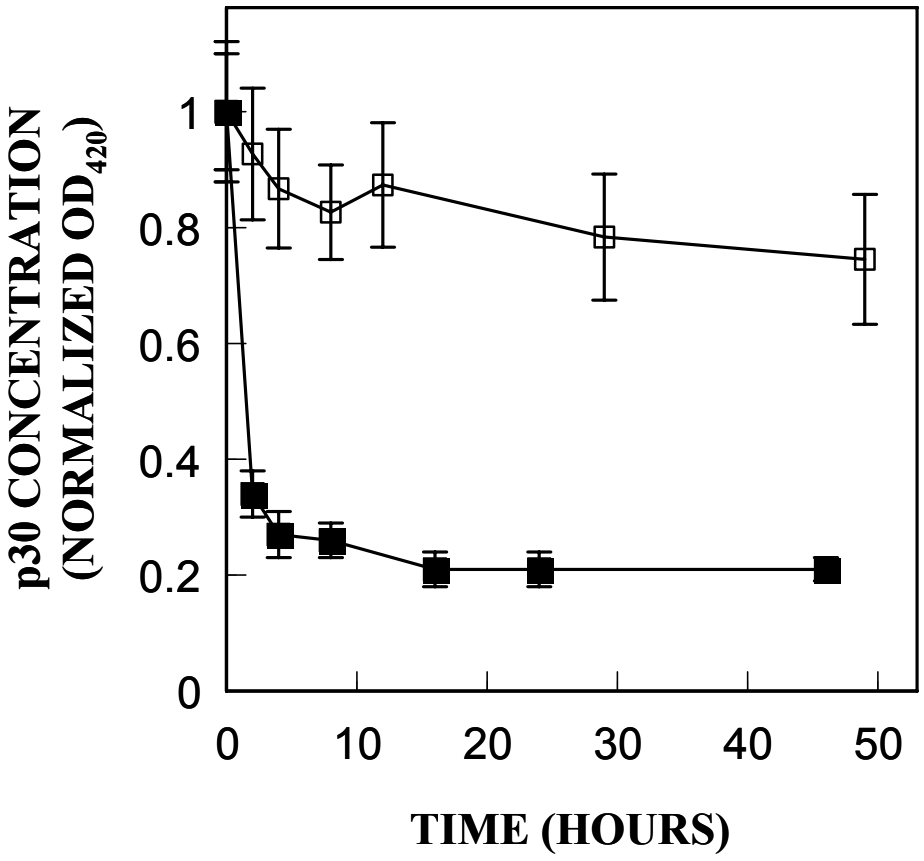

Figure 6.11 Purification by complexation with polymers increases the flux of viruses to cells. Virus stocks produced by TELCeB6-A clone 1-18 were purified by complexation with polymers (■) of by low speed centrifugation (□). Purified stocks were brought to 8 μg/mL of PB, then added to NIH 3T3 cells. At various times after the start of transduction, samples of the supernatant were collected and their concentration of p30 quantified by ELISA. Each point shows the mean ± standard deviation of three replicates. Similar results were obtained with virus stocks produced by other TELCeB6-A clones.

per virus was low (clones 1-18 and 2-17), viruses purified by complexation with polymers achieved higher transduction than viruses purified by low speed centrifugation. However, when the number of envelope proteins was high (clones 2-18 and 2-44), viruses purified by complexation with polymers and viruses purified by low speed centrifugation achieved similar levels of transduction (Figure 6.12). These results indicated that, at high concentrations of virus-associated gp70, increasing the flux of viruses to the surface of the cells did not enhance gene transfer.

6.5 Discussion

In this study we examined how varying the number of envelope proteins per virus particle affects the efficiency of transduction. We found that virus titer, measured by transducing cells with diluted virus stocks, increased as the number of envelope proteins per virus particle increased. In contrast, when transducing cells with undiluted virus stocks, gene transfer was maximal when the number of envelope proteins per virus was low and drastically decreased with increasing numbers of envelope proteins per virus.

Our results from titer are in agreement with previous studies [18] and indicate that individual virus particles displaying high concentrations of gp70 were able to efficiently transduce cells in diluted stocks. However, undiluted stocks contained high levels of free gp70 (refer to Appendix A1) that strongly inhibited infection when applied directly to cells. Free gp70 complicated our efforts to examine the relationship between gene transfer and the number of envelope proteins per virus, so we purified the stocks from this inhibitor using a previously described polymer complexation method (refer to Chapter 5). To examine the relationship between gene transfer and the number of envelope proteins per virus, we used purified virus to transduce NIH 3T3, Hela, and TE671 cells. We were surprised to find that transduction with undiluted and purified stocks displayed a trend opposite to that predicted by titer.

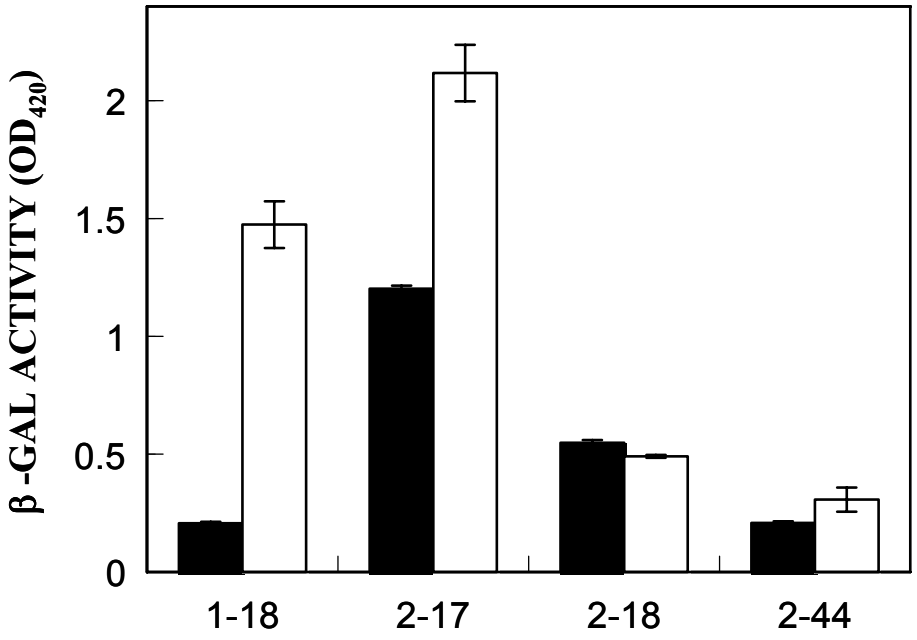

Figure 6.12 At high concentrations of virus-associated gp70, increasing the flux of viruses to cells does not enhance gene transfer. Virus stocks produced by TELCeB6-A clones 1-18, 2-17, 2-18 or 2-44 were purified by complexation with polymers (black bars) of by low speed centrifugation (white bars). Purified stocks were brought to 8 μg/mL of PB, then added to NIH 3T3 cells. Two days later, the efficiency of gene transfer was quantified. Each point shows the mean ± standard deviation of three replicates.

Titer has been commonly used to predict the efficiency of gene transfer in gene therapy protocols. Here we show that titer can be a misleading parameter, as it accounts for the efficiency of transduction with virus stocks that had been diluted several thousand fold in fresh medium, such that each cell is likely to encounter at most one virus particle throughout the transduction experiment. In contrast, during transduction with undiluted stocks, an individual cell may encounter many viruses within a short period of time, which may regulate its susceptibility to transduction or to multiple transduction events.

We found that when cells were transduced with high doses of purified amphotropic virus, further transduction by amphotropic retroviruses, but not ecotropic retroviruses, was blocked. These results suggest that high concentrations of envelope proteins that are associated with a virus particle block or otherwise prevent cell surface receptors from interacting with subsequent virus particles that are pseudotyped with the same envelope protein. We did not examine the specific mechanism by which the receptor-envelope interaction was compromised, but possible mechanisms to consider include saturation and inactivation of cell receptors.

Saturation of cell receptors may occur because each individual virus particle may bind to more receptors than necessary for transduction. A virus particle requires several envelope-receptor interactions in order to mediate efficient fusion. Envelope proteins form oligomers and cooperatively bind to cell receptors [22, 23]. Given that receptors also form oligomers and appear to mobilize and create multiple binding sites for a virus particle [26, 36], it is possible that more receptors than required for fusion colocalize at the site of binding of a virus. Considering this setup, when envelope proteins of a single virus are in excess, more envelope-receptor interactions than those strictly necessary for fusion may take place. In a previous study, we assumed that each retrovirus in a stock produced by pooled clones of TELCeB6-A contained about 200-400 envelope proteins, in analogy with human immunodeficiency virus type 1 [18, 37-39]. Based on this

assumption, we estimated that about 10^5 envelope proteins were delivered per cell (refer to Chapter 5). In the present study, the number of number of envelope proteins per virus varied by more than 20-fold, yet the titers of the stocks remained high, on the order of 10^6-10^7 CFU/mL (Table 6.1). If we consider that an infectious virus from a stock with low concentrations of gp70 displays 200 envelope proteins, we could estimate that 10^6 or more envelope proteins were delivered per cell when stocks with the highest concentrations of gp70 were used. Given that NIH 3T3 cells express about 10^5 amphotropic receptors per cell [40], these approximate calculations suggest that cellular receptors could be saturated after binding to only few viruses.

Upon binding to gp70, receptors on the cell surface internalize and colocalize with gp70 in intracellular compartments [41, 42]. Replenishment of receptors on the cell surface can take several hours, as it has been shown that receptor interference can last 24-48 hours in hematopoietic stem cells [16, 28]. Given that retroviruses decay rapidly, with a half life between 6 and 8 hours [43], it is possible that by the time receptors are replenished at the cell surface, the viruses remaining in the medium had lost their ability to transduce.

In addition to saturation, it is possible that receptors are deactivated and become unable to mediate virus fusion. The amphotropic receptor is a sodium-dependent phosphate transporter [44, 45] and its level of expression is likely regulated to maintain homeostasis. In the presence of high extracellular concentrations of phosphate, receptors remain present at the surface of the cell, yet are deactivated and not able to mediate phosphate uptake or viral transduction [46, 47]. Conversely, in the presence of low concentrations of phosphate, receptors are activated [46, 48]. This activation-deactivation pattern in response to the extracellular phosphate is determined by changes in the structural organization of the receptors and by changes in the organization of the actin cytoskeleton of the target cells [46]. It is possible that high concentrations of gp70

are sensed in manner analogous to that of high concentrations of phosphate, and trigger deactivation of receptors.

We speculate that varying the concentration of virus-associated gp70 can be used to increase the efficiency of transduction or to limit the number of transduction events per target cell. A major concern in the field of gene therapy is that proviral genes can integrate within sequences of functional genes and lead to major physiological disorders [1]. Restricting the number of integration events by increasing the concentration of virus-associated gp70 could decrease the probability of insertional mutagenesis. However, we should consider that our results were obtained on NIH 3T3 cells, which express high concentrations of amphotropic receptors and are easy to transduce. Cell lines with lower levels of receptors could benefit from lower expression of gp70, as multiple fusion events may be necessary before a single integration occurs.

The number of envelope proteins per virus that is suitable for a given application appears to depend on several factors, such as the type of target cells, the concentration of viruses in the stock, the method used to purify or concentrate the stock, and the flux of viruses to the cells. The concentration of virus-associated gp70 that achieved maximal transduction was different for NIH 3T3, TE671 and Hela cells. These differences can be due to variability in the number and regulation of receptors on the surface of the particular target cell. Also, it is possible that receptors from different cells mobilize more or less efficiently to the binding site of a virus or that the efficiency of post-binding steps of transduction varies depending on the cell.

The concentration of viruses and the method used to process a virus stock can also determine the outcome of transduction. Processing of virus stocks is commonly used to enhance gene transfer and usually encompasses purification and concentration procedures [34, 49-54]. In our system, concentration of the stocks might have not increased gene transfer, as maximal transduction was achieved with diluted stocks. On

the contrary, purification increased transduction by eliminating a major inhibitor, namely free gp70. Given that processing of the stocks usually results in loss of a fraction of the viruses, the need to concentrate and purify should be carefully evaluated.

The efficiency of gene transfer is also dependent on the flux of viruses to target cells [34]. Viruses lose their ability to transduce with a short half-life (6-8 hours), and their transport is slow, mainly driven by diffusion [35, 43, 55]. As a result, most viruses lose their ability to transduce before reaching the target cells. Increasing the rate of transport of viruses overcomes this limitation. It enables more viruses to bind to the surface of the cells before losing their bioactivity, thereby increasing the efficiency of transduction. However, in this study we showed that increasing the flux of viruses only enhanced gene transfer when the number of envelope proteins per virus was low. This suggests that methods to accelerate the transport of viruses may not always be necessary.

In summary, the results from this study highlight the importance of using accurate parameters to predict and control the efficiency of gene transfer. We propose that one of these parameters is the number of envelope proteins per virus particle. In order to use it appropriately, we need to better understand the mechanism by which gp70 controls the efficiency of transduction. In light of this, future studies should focus on characterizing the behavior of cell receptors with varying concentrations of viral envelope proteins.

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CHAPTER 7

CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

7.1 Summary of results

In chapter two, we developed a method that substantially enhances the efficiency of retroviral-mediated gene transfer. The method consists of the combined addition of equal weight concentrations of the cationic polymer Polybrene and the anionic polymer chondroitin sulfate C to retrovirus stocks prior to transduction. We found that these polymers aggregate with virus particles and form high molecular weight retrovirus-polymer complexes that can be pelleted by low speed centrifugation. The pelleted complexes contained most of the virus particles, but few other proteins. We were surprised to find that the viruses in the complexes remained bioactive and could be used to transduce cells. When we concentrated the virus stocks by resuspending the virus-polymer complexes in one-eighth the original volume, gene transfer to NIH 3T3 murine fibroblasts increased 10 to 20-fold over the original retrovirus stock that had not been concentrated.

While conducting these experiments, we found that polymers enhanced gene transfer even when the stocks were not pelleted and concentrated. In addition, we observed granular precipitates on the surface of the target cells, which suggested that the complexes were sedimenting onto the cells.

In chapter three, we investigated the mechanism by which the polymers enhance gene transfer without the need for concentration. We found that addition of oppositely charged polymers (Polybrene and chondroitin sulfate C) to virus stocks enhanced gene transfer by increasing the flux of active viruses to the cells. Virus-polymer complexes

formed that did not reduce the stability of the viruses yet were large enough to sediment, delivering the viruses to the cells more rapidly than by simple diffusion. The size of the complexes, the rate of sedimentation, and the levels of gene transfer increased with increasing concentrations of polymers. Interestingly, we found that association of the viruses with the polymer complexes did not hinder their ability to complete post-binding steps of transduction.

In chapter four, we investigated the possibility of flocculating viruses using different combinations of charged polymers. For each combination we tested, viruses associated with polyelectrolyte complexes and significantly enhanced transduction. The anionic polymer and the viruses appeared to bind to most of the cationic polymer, thereby neutralizing the excess of positive charge in the medium. Based on these findings and the observation that viruses are located mainly at the surface of the complexes, we suggested a mechanism for flocculation. We proposed that oppositely charged polymers aggregate and form hydrophobic cores. When the cationic polymer is added in excess of the anionic polymer, molecules of the polycation are displayed at the surface of theses cores, and serve as anchors for binding of viruses to the complexes.

While investigating the mechanism by which combinations of oppositely charged polymers flocculate viruses, we found that it is possible to flocculate and concentrate viruses using a single species of cationic polymer. Viruses concentrated after double flocculation remained as part of polymer complexes and were rapidly transported to the cells. In contrast, viruses concentrated after single flocculation disaggregated from the complexes and behaved as free viruses. We concluded that concentration by flocculation with a single polymer species is an effective alternative for applications for which high concentrations of polymers are undesirable or for which higher rates of virus adsorption due to sedimentation are not necessary.

In chapter five, we examined the possibility of purifying stocks of retroviruses from inhibitors of transduction by inducing formation of virus-polymer complexes, pelleting the complexes and resuspending the pellet to its original volume in fresh cell culture medium. We used virus stocks that contained high levels of inhibitory activity that was most likely due to free viral envelope protein. We found that the pelleted complexes incorporated more than 70% of the virus particles but less than 0.4% of all other proteins and no detectable inhibitory activity. Purified virus transduced cells up to 186-fold more efficiently than virus that was not purified. Interestingly, when high doses of purified virus were used, gene transfer reached a maximum plateau level, most likely because particle-associated amphotropic envelope proteins had saturated the cellular receptors for the virus.

In chapter six, we studied the relationship between the number of amphotropic envelope proteins associated per virus particle and the efficiency of transduction. We produced virus stocks that differed only in their concentrations of envelope proteins. Given that stocks containing high concentrations of virus-associated envelope protein also contained high concentrations of free envelope inhibitor, we purified the stocks by complexation with charged polymers. Using purified stocks, we found that low concentrations of virus-associated envelope achieved the highest efficiencies of transduction, most likely by allowing multiple integrations of the transgene per target cell. In contrast, high concentrations of virus-associated amphotropic envelope inhibited transduction in a receptor dependent manner.

7.2 Conclusions

1) Aggregation of viruses into complexes of oppositely charged polymers dramatically increases the efficiency of retroviral-mediated gene transfer by overcoming major barriers of transduction. Virus-polymer complexes sediment, increasing the

number of active viruses that are delivered to the cells, and are easily concentrated and purified by low speed centrifugation and resuspension in fresh medium (refer to Appendix A2 for a detailed protocol of this method).

2) It is possible to concentrate virus stocks by inducing aggregation of viruses with a single species of cationic polymer, while only incorporating trace amounts of the polymer to the processed stock. Viruses concentrated by this means are subject to diffusion limitations, but can be effectively used in applications where an excess of polymers is undesirable. It is important to note that more research is necessary to characterize flocculation with a single polymer for its ability to purify viruses from inhibitors of transduction.

3) Purified virus stocks can be used in smaller quantities than unprocessed stocks to achieve a desired level of gene transfer. Also, purification reduces uncertainties about the relationship between the amount of virus used and the number of genes transferred, which should simplify efforts to predict the outcome of retrovirus transduction protocols.

4) The efficiency of gene transfer, and potentially the number of transgene copies delivered per target cell, can be modulated by varying the number of envelope proteins that protrude from a virus particle.

7.3 Suggestions for future research

1) Given that double flocculation of viruses with oppositely charged polymers is a promising means to overcome major extracellular barriers of retroviral-mediated gene transfer, future research should focus on determining the effect that high concentrations of charged polymers may have on target cells *ex vivo* and *in vivo*. In our model system, polymers were not toxic, but their applicability to more sensitive cells or directly *in vivo* has not been evaluated. Moreover, it is not clear if viruses

disaggregate from the polyelectrolyte complexes before fusing with the target cells or if the polymers are co-internalized with the viruses.

2) In order to better understand why viruses are selectively incorporated into polyelectrolyte complexes and how viruses within complexes can efficiently accomplish post-binding steps of transduction, it is necessary to characterize the physicochemical properties of the virus-polymer aggregates. Future research should focus on quantifying the size, charge and composition of the complexes. In addition, future research should examine the kinetics of flocculation and the stability of the complexes depending on the medium they are suspended in.

3) Flocculation with a single species of cationic polymer appears as an effective alternative to dual flocculation for concentrating virus stocks, which minimizes the concentration of polymers that remain associated with virus particles. Future research should examine the selectivity of a single polymer for aggregating virus particles and not other components of the medium. More specifically, it should test the possibility of purifying virus stocks from inhibitors or undesirable impurities by single flocculation.

4) We developed and evaluated a method for concentrating and purifying stocks of retroviruses and lentiviruses and we showed it is rapid, easy and efficient at small scale. Future research should evaluate dual flocculation and single flocculation as methods for large scale processing of virus stocks.

5) Flocculation of retroviruses with polymers can have a wide variety of applications, which should be investigated in the future. For example, flocculation could be used to detect the presence of viruses in different media (e.g. in blood serum).

6) Our results indicate that it is possible to enhance gene transfer by regulating the number of envelope proteins per virus particle. Given that these results were based on transduction of cells that express high concentrations of receptors, future

research should examine the possibility of enhancing gene transfer by this means to cells that express low concentrations of receptors and are difficult to transduce (e.g stem cells).

7) Our results suggest that it may be possible to restrict the number of transgene copies delivered per target cell by increasing the number of envelope proteins per virus particle. Since limiting the number of transgenes per cell can reduce the risk of insertional mutagenesis, future research should further explore this possibility using target cells that are relevant to gene therapies.

8) Our results indicate that when the number of envelope proteins per virus particle is high, transduction is limited due to a reduction in availability of functional receptors for viruses pseudotyped with the same envelope. Future research should investigate the mechanism by which receptors block transduction in response to high concentrations of virus-associated gp70. A better understanding of this mechanism would permit developing therapies to block infection or strategies to increase the efficiency of transduction.

APPENDIX A1

THE CONCENTRATION OF FREE ENVELOPE PROTEINS IS PROPORTIONAL TO THE CONCENTRATION OF ENVELOPE PROTEINS ASSOCIATED WITH VIRUS PARTICLES

The total number of viral envelope proteins (gp70) present in a virus stock corresponds to the sum of envelope proteins that are not associated with viruses (free gp70) and envelope proteins that protrude from the surface of virus particles (virus-associated gp70). In this section, we examined the relationship between the total number of gp70, free gp70 and virus-associated gp70.

In Chapter 6, we constructed a panel of several clonal amphotropic murine leukemia virus packaging cell lines, each of which was derived from the same parental cell line (TELCeB6 cells). These packaging cell lines (TELCeB6-A) differed only in the amount of envelope protein they expressed. We produced virus stocks from these clonal cell lines and measured the fraction of gp70 that was free or that was associated with viruses in each stock using two different methods: (1) ultracentrifugation and (2) complexation of viruses with polymers.

Ultracentrifugation. To determine how the concentration of free gp70 varied with varying concentrations of total gp70, 9 mL of virus stocks were centrifuged over 2 mL of 20% sucrose cushion for 90 min at 40000 rpm (200000g) and 4°C. Eight milliliters of the supernatant were collected. Upon ultracentrifugation, the virus capsid protein was not detected in the supernatant (data not shown), indicating that all the particles had been pelleted. However, the concentration of free gp70 remaining in the supernatant increased with increasing concentrations of gp70 in the original virus stock (Figure A2.1).

This correlation appeared to be linear (R^2 = 0.89) and, by regression, we estimated that 27 $\pm$ 5% (P = 0.005) of the total gp70 in the stocks was free.

Complexation of viruses with polymers. In Chapter 5, we showed that virus stocks could be purified from free gp70 by inducing association of virus particles into polymer complexes. Briefly, stocks were brought to equal weight concentrations (80 μg/mL) of PB and CSC by adding, in immediate succession, appropriate volumes of aqueous stock solutions (20 mg/mL) of the polymers and vortexing the mixture. The mixture was incubated for 20 min at 37°C, then centrifuged at 10000g for 5 min. The supernatant was decanted and the pellet was resuspended to the original volume in fresh cell culture medium. We purified virus stocks produced by TELCeB6-A clones using this procedure, then compared the concentrations of viral capsid protein (p30) and gp70 in the original and the purified stocks. The pellet concentrated 87% (R^2 = 0.77) of the original content of p30 (Figure A2.2). Differences in expression of p30 from stock to stock were most likely due to differences in the confluency of packaging cells when the virus was harvested. The concentration of virus-associated gp70 in the resuspended pellet directly correlated with the concentration of total gp70 in the original stock (Figure A2.3). Assuming a linear correlation (R^2 = 0.89), we estimated that 67 $\pm$ 4% (P = 0.000) of gp70 was associated with virus particles, which is consistent with the results from ultracentrifugation.

We concluded that the average number of free envelope proteins was proportional to the number of envelope proteins incorporated per virus particle and to the amount of envelope protein that was expressed by the cells that produced them. In our system, about 30% of the envelope proteins in each virus stock was free gp70.

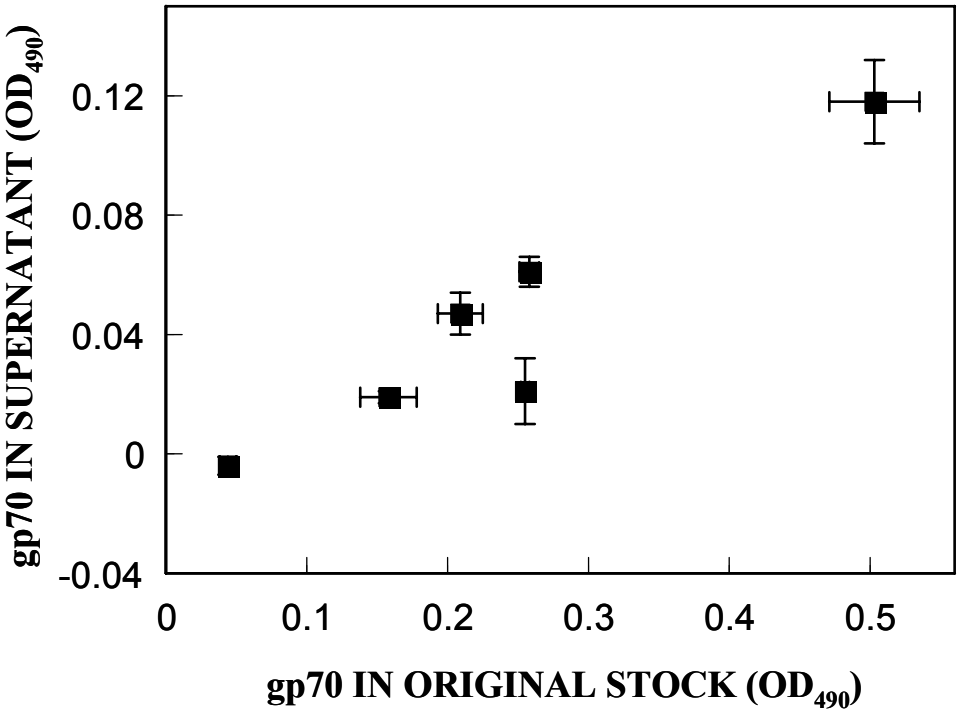

Figure A1.1 The concentration of free gp70 in a virus stock is proportional to the concentration of virus-associated gp70. Virus stocks produced by various TELCeB6-A clones were ultracentrifuged, then the concentration of gp70 in the original stock and in the supernatant (free gp70) were measured by ELISA. Each point shows the mean ± standard deviation of three wells.

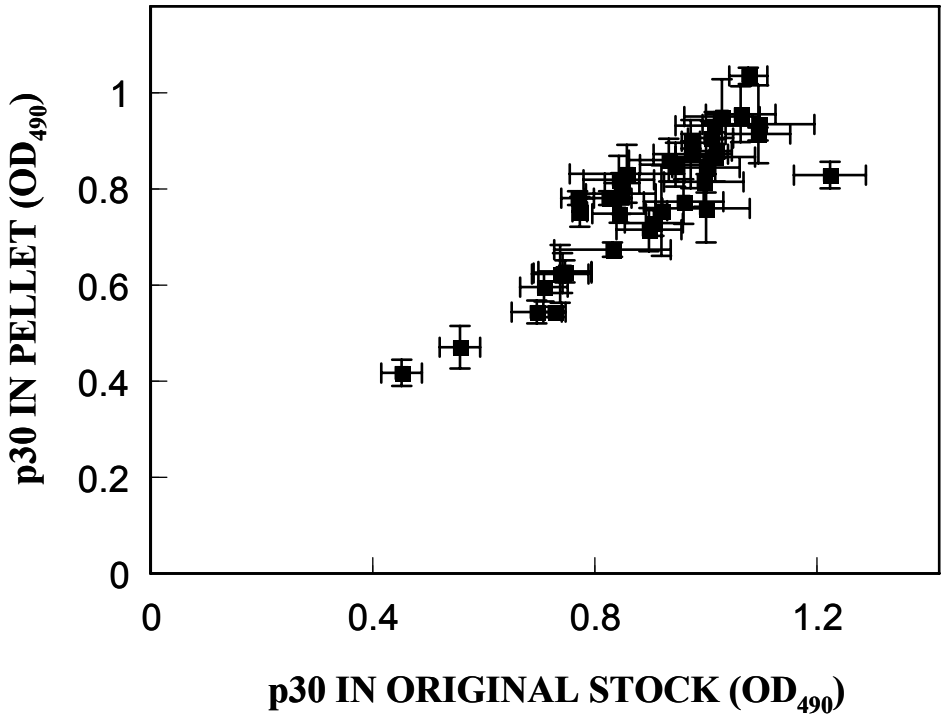

Figure A1.2 Recovery of p30 upon purification by complexation with polymers. Virus stocks produced by various TELCeB6-A clones were brought to 80 μg/mL of PB and CSC, incubated at 37°C for 20 min, then centrifuged for 5 min at 10000g. The pellets were resuspended to the original volume in fresh medium. The concentrations of p30 in the original stocks and the resuspended pellets were measured by ELISA. Each point shows the mean ± standard deviation of three replicates.

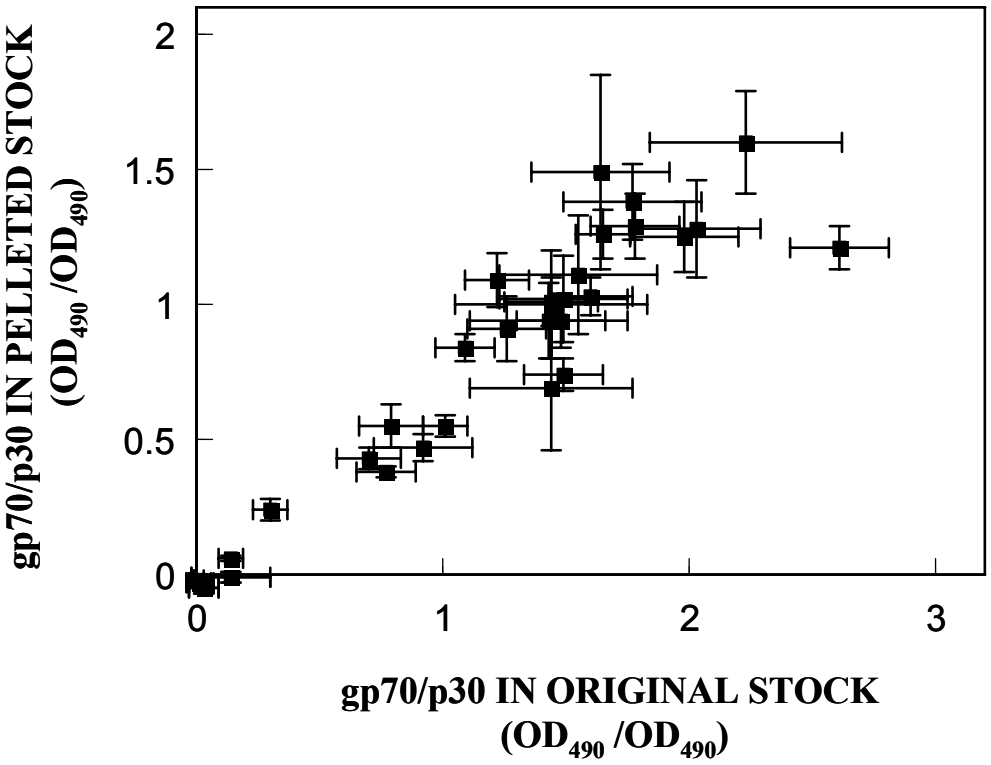

Figure A1.3 The concentration of virus-associated gp70 in purified stocks is proportional to the total concentration of gp70 in original stocks. Virus stocks produced by various TELCeB6-A clones were brought to 80 μg/mL of PB and CSC, incubated at 37°C for 20 min, then centrifuged for 5 min at 10000g. The pellets were resuspended to the original volume in fresh medium. The concentrations of gp70 in the original stocks and the resuspended pellets were measured by ELISA. The concentrations of gp70 were normalized with the concentrations of p30. Each point shows the mean ± standard deviation of three replicates.

APPENDIX A2

PROTOCOL FOR RETROVIRUS / LENTIVIRUS CONCENTRATION, PURIFICATION, AND DELIVERY USING VIRUS-POLYMER COMPLEXES

Materials (polymers)

1. Chondroitin sulfate C (CSC): Chondroitin 6-sulfate sodium salt from shark cartilage (Sigma-Aldrich, catalog # C4384)
2. Polybrene (PB): Hexadimethrine bromide (Sigma-Aldrich, catalog # H9268)

Procedure

A. Preparation of stock solutions of PB and CSC (250X concentrate)

1. Prepare separate stock solutions for each polymer. Dissolve each polymer in distilled water (20 mg/mL), then filter-sterilize it using a 0.45 μm-pore filter. Store these stock solutions at 4°C.

   We recommend that fresh stock solutions be prepared each month. Use of stock solutions older than one month may lead to lower recoveries of virus and lower levels of gene transfer.

B. Concentration and purification of retrovirus or lentivirus stocks

1. Bring the stock of retrovirus (or lentivirus) to 80 μg/mL of PB and 80 μg/mL of CSC by adding, in immediate succession, appropriate volumes of the stock solutions of PB and CSC (1/250th of the final volume) to the virus stock. Mix by vortexing quickly or by pipetting up and down.

Polymer concentrations have been optimized for stocks of amphotropic retrovirus produced by the ψ-CRIP packaging cell line (derived from NIH 3T3) and the TELCeB6 packaging cell line (derived from TE671). Equal weight concentrations of both polymers have been shown to achieve optimal gene transfer [1]. We recommend that new users determine the optimum concentration of polymers for their experimental system by measuring the dose response of gene transfer as a function of polymer concentration while maintaining a 1:1 ratio of PB:CSC (see section D for sample optimization protocol).

2. Incubate this solution at 37°C for 20 min. During this time PB and CSC aggregate with virus particles and form virus-polymer complexes.

   We have found that incubation times of 5 to 20 minutes work well with amphotropic retrovirus stocks produced by the ψ-CRIP packaging cell line. The optimum time required to incubate viruses with polymers to form virus-polymer complexes may vary among different virus production systems. Most likely, 20 minutes will be more than adequate for most systems. The incubation time is to ensure that as many virus particles as possible have become associated with polymer complexes before the complexes are centrifuged and resuspended in fresh medium.

   Do not allow the virus stock to become too basic (pH > 7.8) because retrovirus stability declines rapidly if the pH becomes too high.. This should not be a problem as long as incubation times are kept short (not significantly longer than 20 min).

3. Centrifuge the solution at 10,000g for 5 min. A visible pellet (the virus-polymer complexes) should be visible at the bottom of the tube.

Under these conditions, which have been optimized for the centrifugation of virus in microcentrifuge tubes (1.5 mL) using a table-top centrifuge (e.g., Eppendorf 5417C), we routinely recover 80% of the virus particles. It is possible that the optimum centrifugation speeds and times may vary if larger volumes of virus are centrifuged, or if different rotors or centrifuges are used.

4. Remove the supernatant from the pellet.

   When using microcentrifuge tubes, the pellet is compact and firmly adherent to the side of the tube, but could detach if agitated. To aspirate the supernatant, tilt the tube to avoid aspirating the pellet or touching the pellet with the tip of the pipette or aspirator. If necessary, re-centrifuge to re-form the pellet at the bottom of the tube..

5. Add fresh cell culture medium to the pellet. Resuspend the pellet by pipetting up and down until visible aggregates disappear.

   The volume of fresh medium that is added to the pellet depends on the fold-concentration desired. For example, to concentrate 1 mL of virus 10-fold, pellet the virus, then resuspend in 100 μL of fresh medium. To purify 1 mL of virus and/or resuspend it in a new type of cell culture medium without concentrating it, resuspend the pellet with fresh medium to its original volume (1 mL).

   When using microcentrifuge tubes, it is easier to resuspend the pellet using a small diameter pipette tip and small volumes of resuspension medium (50-100 μL) to help break up the pellet. If you wish to resuspend the pellet to a larger volume, it is helpful to first resuspend the pellet in a small volume (50-100 μL) until the pellet is completely resuspended before bringing the virus to its final volume.

To determine the percentage recovery of virus particles in the pellet, resuspend the pellet to the original volume in fresh medium, then measure the concentration of viral capsid protein by ELISA and compare it to that of the original stock solution. Measurements of titer increase several fold [1] when using the resuspended pellet and therefore cannot be used to accurately assess the percentage recovery of virus particles.

C. Example transduction protocol: Transduction of NIH 3T3 murine fibroblasts with virus-polymer complexes

Day 1

1. Plate 5000 NIH 3T3 cells per well in a 96-well plate. Incubate the plate overnight at 37°C and 10% CO_2.

Day 2

1. Prepare virus stocks using the concentration/purification procedure:
    i. To purify the virus without concentrating it, bring 400 μL of virus stock in a microcentrifuge tube to equal weight concentrations of PB and CSC. Follow steps B2 to B5 and resuspend the pellet to its original volume in fresh medium.

    We generally bring the concentration of PB and CSC to 80 μg/mL; however, new users should determine the polymer concentrations that are optimal in their hands; in addition, optimum polymer concentrations should also be determined any time a new cell type or virus is used.

    ii. To concentrate the virus 8-fold, bring (2) 1480 μL volumes of virus stock in microcentrifuge tubes to equal weight concentrations of PB and CSC.

Follow steps B2 to B5. Resuspend each pellet in 185 μL of fresh medium, then combine into a single tube for a total volume of 370 μL of concentrated virus-polymer complexes.

We generally bring the concentration of PB and CSC to 80 μg/mL; however, the optimal concentration of polymers used should be determined for each new virus-cell system as described below. It is also important to determine to what extent the virus should be concentrated in order to obtain the highest levels of gene transfer.

2. Carefully remove the medium from the cells by aspiration or by inverting the plate over sterile paper towels.
3. Add 100 μL per well of the virus-polymer complexes to triplicate wells. As a control, add 100 μL per well of untreated virus stock to triplicate wells. Add fresh medium to the rest of the wells.
4. Incubate the plate for two days at 37°C and 10% CO_2.

Precipitates will be visible on the surface of the target cells within a few minutes after the start of transduction and will accumulate over several hours. These structures are virus-polymer complexes that have sedimented onto the surfaces of the cells [1, 2]. The presence of virus-polymer complexes on cells has been shown to <u>not</u> be cytotoxic for a number of target cell lines (including NIH 3T3, Hela, RIE) and to <u>not</u> interfere with post-binding steps of gene transfer [2]. Nevertheless, we recommend that appropriate control experiments are conducted to verify that the virus-polymer complexes do not adversely affect cell function.

<u>Day 4</u>

Quantify the efficiency of gene transfer by measuring the level of β-galactosidase activity in the lysates of the transduced cells.

D. Optimization of polymer concentrations

1. Add equal weight concentrations of PB and CSC ranging from 20 to 320 μg/mL of both polymers to stocks of retrovirus. For example, add 20, 40, 80, 120, 160, and 320 μg/mL of both polymers to 400 μl of virus stock in microcentrifuge tubes.
2. Follow steps B2 to B4 for each tube, then resuspend each pellet in 400 μl (the original volume) of fresh medium.
3. Transduce NIH 3T3 target cells plated the previous day in 96-well plates by adding 100 μl per well of each resuspended pellet to triplicate wells. As a control, add 100 μl per well of untreated virus stock. (see the part C for additional details).
4. Two days later, measure the level of β-Gal activity in lysates of transduced cells [1]. Plot β-Gal activity (which is proportional to the number of genes transferred) as a function of the concentration of PB and CSC that was added to the virus stock. Determine what polymer concentration results in the highest levels of gene transfer (usually in the range of 40 to 120 μg/mL).